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P2X Purinergic Receptor Knockout Mice Reveal Endogenous ATP Modulation of Both AVP and OT Release from the Intact Neurohypophysis

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Abstract

Bursts of action potentials are crucial for neuropeptide release from the Hypothalamic Neurohypophysial System (HNS). The biophysical properties of the ion channels involved in release of these neuropeptides cannot explain the efficacy of such bursting patterns on secretion. We have previously shown that ATP, acting via P2X receptors, potentiates only AVP release from HNS terminals, whereas, its metabolite adenosine, via A1 receptors acting on transient Ca^{2+} currents, inhibits both AVP and OT secretion. Thus, purinergic feedback-mechanisms have been proposed to explain bursting efficacy at HNS terminals.

Therefore, in the present study we have used specific P2X receptor knockout (rKO) mice and purportedly selective P2X receptor antagonists to determine the P2X receptor subtype responsible for endogenous ATP induced potentiation of electrically stimulated neuropeptide release. Intact neurohypophyses (NH) from wild type (WT), P2X3 rKO, P2X2&3 rKO and P2X7 rKO mice were electrically stimulated with four 25 second bursts (3V at 39Hz) separated by 21 second interburst intervals with or without the P2X2 and P2X3 receptor antagonists, suramin or PPADS. These frequencies, number of bursts, and voltages were determined to maximize both AVP and OT release by electrical stimulations.

Treatment of WT mouse NH with suramin/PPADS significantly reduced electrically stimulated AVP release. A similar inhibition by suramin was observed in electrically stimulated NH from P2X3 and P2X7 rKO mice but not P2X2&3 rKO mice, indicating that endogenous ATP facilitation of electrically stimulated AVP release is mediated primarily by the activation of the P2X2 receptor. Surprisingly, electrically stimulated OT release from WT, P2X3, P2X2&3 and P2X7 rKO mice was potentiated by suramin, indicating non-purinergic effects by this “selective” antagonist. Nevertheless, these results show that sufficient endogenous ATP is released by bursts of action potentials to act at P2X2 receptors in a positive-feedback mechanism to differentially modulate neuropeptide release from CNS terminals.

Keywords

neuropeptides; oxytocin; vasopressin; P2X receptors; Knock outs

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Introduction

Bursts of action potentials are crucial for release of most neurotransmitters from dense core granules. This has been most definitively shown for neuropeptide release [1] from the Hypothalamic Neurohypophyseal System (HNS). Why different bursting patterns for the two neuropeptides are necessary, however, is not well understood. The biophysical properties of the ion channels involved in release of these neuropeptides cannot explain the efficacy of such bursting patterns on secretion. That is, although there are different calcium channels in vasopressin (AVP) *vs.* oxytocin (OT) terminals [2], their activation/inactivation properties by themselves do not explain the differential effects of bursting [3].

We have previously shown that ATP, acting via P2X receptors, potentiates only AVP release from HNS terminals [4], whereas, its metabolite adenosine, via A1 receptors acting on transient Ca^{2+} currents, inhibits both AVP and OT secretion [5]. We have shown that exogenous ATP induces inward currents [6] and causes release of only vasopressin (AVP) from neurohypophyseal terminals [4]; both effects are inhibited by suramin and PPADS, selective, but not specific, P2X2 and P2X3 receptor antagonists. Recently, we determined, in the rat neurohypophysis, that endogenous ATP levels were sufficient to facilitate depolarization induced AVP release [7]. Furthermore, using IR we have identified P2X2, 3, 4 & 7R in AVP-terminals, but only P2X7R in OT-terminals [6]. Thus, purinergic feedback-mechanisms have been proposed to explain this potentiation of AVP release during bursts at HNS terminals (see Figure 7).

Therefore, the objectives of this study were to determine: 1) if endogenous ATP levels are sufficient to cause tonic modulation of neuropeptide release stimulated by bursts of action potentials in the **mouse** HNS and 2) using specific P2X receptor knockout mice determine the P2X receptor type responsible for the tonic modulation of neuropeptide release. These rKO mice are superior to the not-very selective P2X antagonists currently available. We could, thus, test our model (see Figure 7) and **hypotheses** that ATP co-released with the peptides would specifically potentiate subsequent AVP release at NH terminals. Breakdown into adenosine, by ectonucleotidases, would eventually inhibit release of both peptides. These dual, conflicting effects of endogenous ATP would, thus, help to explain the patterns of AVP release observed during physiological stimulation in vivo. OT release, in contrast, without this potentiation by ATP, would need higher frequency stimulation for facilitated release [8].

Methods

Isolation and Electrical Stimulation of the Neurohypophysis

Male Sprague-Dawley CD rats or male C57BL mice (Taconic Farms, Germantown, NY) were immobilized and sacrificed by decapitation with a guillotine, following the guidelines laid down by the UMMS ethical committee, and the pituitary isolated. Following removal of the anterior and intermediate lobes, each posterior pituitary (neurohypophysis) was hooked onto a platinum wire electrode in a plastic perfusion chamber [1] and was perfused at 125 $\mu\text{L}/\text{minute}$ at 37°C with a modified Normal Locke's saline containing (in mM): NaCl 140; KCl 2.5; CaCl_2 1.2; HEPES 10; Glucose 7; MgCl_2 .3; pH 7.55 and the osmolality was 295–300 mOsmol/L. After an hour long equilibration period, each neurohypophysis was electrically stimulated using biphasic 2 ms duration pulses passing a current of $\sim 20 \mu\text{A}$. Each burst lasted for 25 seconds and when more than one burst was given there was a 21 second rest interval between each burst. The frequency within each burst ranged from 7 to 100Hz. In each experiment using the selective P2X2 and P2X3 receptor antagonists, suramin or PPADS, each neurohypophysis was electrically stimulated 3 times with at least a forty minute rest period between stimulations. The first stimulation (S1) served as a control

for the stimulation in the presence of the selective P2X2 and P2X3 receptor antagonists, suramin or PPADS (S2). Suramin (5, 10 or 15 μ M) or PPADS (5 μ M) were perfused for 16 minutes before, during and for 16 minutes after the second electrical stimulation. The third electrical stimulation was used to determine recovery following removal of the suramin or PPADS. Perfusate samples were collected at 2 to 4-minute intervals during each experiment. Basal release was determined by averaging three to four samples prior to electrical stimulation and was subtracted from each sample during the electrical stimulation to calculate evoked release. The S2/S1 ratio was utilized to determine any effect of the selective P2X2 and P2X3 antagonists, suramin or PPADS, on evoked release of AVP or OT. AVP and OT content in each sample were determined using a specific Enzyme-linked Immunoassay Kit (Assay Designs, Ann Arbor, MI). The assay sensitivity was 0.25 pg/tube for AVP and 1.5 pg/tube for OT [9].

Materials

The P2X3 [10] and P2X2&3 [11] receptor knockout mice were generously provided by Dr. Debra Cockayne (Roche Palo Alto, CA). P2X7 rKO mice were purchased from Jackson labs (Bar Harbor, Maine). The selective P2X2 and P2X3 receptor antagonists suramin and Pyridoxal-phosphate-6-azophenyl-2',4'-disulfonic acid (PPADS) were purchased from Sigma-Aldrich (Saint Louis, MO).

Statistical Analysis

Baseline concentrations of AVP and OT were determined as the average release in the 4 samples collected prior to electrical stimulation of the neurohypophysis. Evoked release of AVP and OT during electrical stimulation of the NH was determined by subtracting the peak amplitude of the response from the mean baseline concentration. Three concentrations of suramin were used (5, 10 and 15 μ M) and each concentration had the same inhibitory effect on electrically stimulated AVP release and potentiating effect on OT release, therefore the data for each concentration of suramin within each treatment group were pooled for statistical analysis. The effect of suramin or PPADS on basal and electrically stimulated (S2/S1) AVP and OT release was determined by analysis of variance for more than two treatment groups or by paired t-test when determining the effect of suramin or PPADS within individual glands using the computer based program Sigma Stat (Jandel Scientific, San Rafael CA). Tukey's HSD test was used to determine differences between means. $P < .05$ was considered statistically significant. Results are expressed as the mean $\pm$ SE.

Results

Effects of Voltage, Number of Bursts, and Frequency on AVP and OT Release

In rat neurohypophysial glands, electrically stimulated (at 39Hz frequency) neuropeptide release increased with increasing voltage that peaked at 3.5V (Figure 1; top panel). In addition, increasing the number of 25 second long bursts also increased the total amount of neuropeptide released during electrical stimulation of the rat neurohypophysis, with the maximum release obtained with a minimum of three bursts (Figure 1; middle panel). Furthermore, as frequency within the burst was increased, electrically stimulated AVP release initially increased, but peaked at 28Hz and then decreased as frequency was further increased to 56 and 100Hz. Interestingly, electrically stimulated OT release peaked at a higher frequency (56Hz) and then started to diminish at 100Hz (Figure 1; bottom panel). Therefore, in order to maximize both AVP and OT release all electrical stimulations in subsequent experiments were given as four bursts at 3V and an intermediate peak frequency of 39Hz with each burst separated by a 21 second interburst interval [1]. The above parameters determined in the rat neurohypophysis were utilized in all subsequent

experiments in mice since Gainer et al., [12] found that the frequency-dependence of AVP secretion from the mouse neurohypophysis in vitro was comparable to that for the rat neurohypophysis in vitro. Therefore, the electrical stimulation paradigm used in the present study, primarily optimized for rat would be comparable in mice.

Effect of P2X2 and P2X3 Receptor Antagonists on Electrically Stimulated AVP and OT Release

The selective P2X2 and P2X3 receptor antagonists, suramin and PPADS, were used to determine if endogenously released ATP contributes to electrically stimulated AVP and/or OT release from the intact neurohypophysis of the mouse. PPADS (5 μ M) and suramin (5 – 15 μ M) at the concentrations utilized in this paper are selective antagonist of the P2X2, P2X3 and P2X2&3 receptor but have no effect on the P2X4 or P2X7 receptor [13,14]. In addition, PPADS concentrations >30 μ M are required to antagonize P2Y receptors, whereas, suramin concentrations used in this paper could have effects on P2Y receptors [15] and G-protein coupled receptors [16]. Thus, using specific receptor KO mice is one of the best options to address the identity of the receptor involved in modulation of release from NH terminals. However, care must be taken in using KO mice, because of possible compensation. Nevertheless, recent studies [17] have shown that there is no compensation by other P2X receptors in purinergic receptor KOs.

Pretreatment of mouse neurohypophysial glands with suramin (5–15 μ M; Figure 2; panel A) or PPADS (5 μ M; Figure 3; panel A) had no effect ($P>.05$) on basal release of AVP, whereas, both suramin (Figure 2; panel B) and PPADS (Figure 3; panel B) increased basal release of OT. Electrically stimulated (4 bursts; 39Hz: 3V) release of AVP was significantly ($P<.05$) decreased ($42.87 \pm 3.24\%$) by suramin (Figure 2; panel A). PPADS had a similar effect on electrically stimulated AVP release (Figure 3; panel A). Surprisingly, suramin (Figure 2; panel B) significantly ($P<.05$) increased electrically stimulated OT release from the mouse neurohypophysial gland by $93.81 \pm 19.10\%$. PPADS had a similar but smaller increase on electrically stimulated OT release (Figure 3, panel B). Summary of effects on mouse neurohypophysial glands by suramin is depicted in Figure 2, panels C and D.

Effect of suramin/PPADS on electrically stimulated AVP and OT Release from Wild Type, P2X3, P2X2&3 and P2X7 Receptor Knockout Mice

P2X3, P2X2&3 and P2X7 receptor knockout mice were used to determine the receptor subtype which contributes to the facilitation of electrically stimulated AVP release by endogenously released ATP from the intact mouse neurohypophysial gland. Suramin (5–15 μ M) had no effect on the basal release of AVP in WT ($n=10$), P2X3 rKO ($n=4$), P2X2&3 rKO ($n=6$) or P2X7 rKO ($n=2$) mice (Figure 4), whereas, suramin increased the basal release of OT in WT, P2X3, P2X2&3 and P2X7 rKO mice (Figure 6). Suramin had the same effect in decreasing ($P>.05$) electrically stimulated AVP release from WT ($48.88 \pm 3.08\%$), P2X3 ($47.05 \pm 3.37\%$) and P2X7 (58%) rKO mice (Figures 4 & 5). However, suramin had significantly ($P<.05$) less of an effect on electrically stimulated AVP release from P2X2&3 rKO mice ($24.23 \pm 10.68\%$) than WT controls ($48.88 \pm 3.08\%$; Figure 5). Suramin had the same effect in potentiating electrically stimulated OT release from WT, P2X3, P2X7 and P2X2&3 rKO mice (Figure 6). PPADS (5 μ M) had no effect on electrically stimulated AVP release from P2X2&3 rKO mice (Figure 3; panel C) but decreased (24%; $n=2$) electrically stimulated AVP release from WT mice (Figure 3; panel D).

Discussion

Isolated neurohypophyses of the rat exhibit voltage- and burst number-dependent release of both AVP and OT (Figure 1). AVP-release exhibits a different frequency dependence than

OT-release (Figure 1), similar to the results reported by Bicknell [18]. Therefore, to maximize both AVP and OT release all electrical stimulations were given as four bursts at 3V and an intermediate peak frequency (39Hz) with each burst separated by a 21 second interburst interval (see [1]). The rat data shown here demonstrates the effectiveness of the stimulation paradigm in our hands. In addition, Gainer et al., [12] found that the frequency-dependence of secretion of AVP from the mouse neural lobe was comparable to that reported for the rat neural lobe. Therefore, we feel that the electrical stimulation paradigm used in the present study, primarily optimized for rat would be comparable in mice.

The data from this study demonstrates that mouse neurohypophysial glands release sufficient endogenous ATP during electrical stimulation to activate PPADS/suramin sensitive P2X receptors resulting in facilitation of AVP but not OT release (Figures 2–4). This was not surprising given that P2X2, P2X3, P2X4 and P2X7 receptors were localized on AVP terminals and only P2X7 receptors were identified on OT terminals [6], and that PPADS (5 μ M) and suramin (5–15 μ M) at the concentrations used are selective P2X2, P2X3 and P2X2&3 receptor antagonist without any effect on P2X4 or P2X7 receptors [13,14]. Therefore the facilitatory effect of endogenous ATP on electrically stimulated AVP release could be due to activation of P2X2, P2X3 and/or P2x2&3 receptors. Surprisingly, suramin, but to a much lesser extent PPADS, potentiated OT release. The effects of suramin on OT release could be due to suramin's effects on G-protein coupled receptors such as the adenosine A1 receptor and the μ -opioid receptor, both of which have been shown to have inhibitory effects on electrically stimulated OT release in the intact rat neurohypophysis.

Since no specific P2X2 and P2X3 receptor antagonist are currently available we combined the use of the selective P2X2 and P2X3 antagonist, PPADS and suramin, with specific P2X receptor knockout mice to ascertain the identity of the P2X receptors involved in the facilitation of electrically stimulated AVP release. The effect of suramin on electrically stimulated AVP release by neurohypophyses from P2X3, and P2X7 receptor knockout mice was similar to that observed in the wild type controls. (Figures 4 & 5). This would indicate that the P2X3 receptor may not contribute to the facilitatory effect of endogenous ATP on AVP release. This interpretation must be viewed with caution since the P2X3 receptor is a high affinity ($EC_{50} < 1\mu$ M) fast desensitizing receptor. In fact it has been reported that endogenously released ATP is sufficient to desensitize P2X3 receptors and P2X2&3 chimeras but not P2X2 receptors [20]. Therefore it is possible that our electrical stimulation paradigm releases endogenous ATP at a concentration sufficient to rapidly desensitize the P2X3 receptor. Suramin had less of an effect on electrically stimulated AVP release from P2X2&3 receptor knockout mice than wild type controls. The fact that suramin's full effect was not overcome in the P2X2&3 receptor knockout mice would indicate that the facilitatory effect on AVP release is not entirely dependent on P2X receptor activation. At the concentrations used in this study suramin would have no effect on the P2X4 or P2X7 receptors which are present on AVP containing terminals. Suramin's effect on inhibitory G-protein coupled receptors such as the adenosine A1 receptor would potentiate AVP release. Adenosine A2A receptors have been immunohistochemically identified in AVP containing terminals and blockade of these G-protein coupled receptors would result in an attenuation of electrically stimulated AVP release, which could account for the effect of suramin in the P2X2&3 knockout mice. In support of this, PPADS, which is not an inhibitor of G-protein coupled receptors, had no effect on electrically stimulated AVP release from P2x2&3 receptor knockout mice but did attenuate AVP release in wild type controls. These data indicate that endogenous ATP facilitation of electrically stimulated AVP release in the mouse neurohypophysis is mediated, at least in part, by activation of the P2X2 receptor (Figures 3 & 7). A role for the P2X3 and P2X2&3 receptor cannot be ruled out. A similar conclusion was recently reported using rat HNS explants [18]. Thus, this justifies our use of

P2X rKO mice to better demonstrate the role of these receptors in physiological release patterns in the HNS.

Future studies using the P2X rKO mice will enable us to determine if our model (see Figure 7) for endogenous ATP feedback effects are valid in the HNS. Nevertheless, we have shown that AVP release is specifically potentiated by endogenous ATP, but OT release is either unaffected or, perhaps, inhibited. These effects appear to be via P2X₂, P2X₃ and/or P2X_{2&3} receptors. These results are consistent with our models of AVP vs. OT burst efficacy [3]. OT neurons are characterized by a high frequency (up to 120 Hz) discharge during suckling which leads to the pulsatile release of OT into the blood and to subsequent milk-ejection [21]. AVP neurons are characterized by their asynchronous (10–60 Hz) phasic activity (bursting) during maintained AVP release and the subsequent regulation of water balance. In both cases, it is the clustering of spikes, albeit with different time courses for each peptide, which facilitates hormone release [1, 22, 23].

Many systems, in particular peptide-releasing neurons, maximally release transmitter only in response to specific “bursting” patterns of activity [1, 21, 24, 25, 26]. This is probably because in most, if not all, peptidergic neurons, including NH terminals [27], there are **no** specific release sites. This means that bulk intracellular $[Ca^{2+}]$ must be elevated sufficiently throughout the terminal in order for release of the peptides to occur. Entry through voltage-gated Ca-channels during one action potential is not enough to reach such levels [28] and thus the need for trains of action potentials and, we now speculate, the added Ca-entry through ATP receptors at terminals. It has also been reported that ATP is stored at high concentrations in most secretory vesicles and is released from chromaffin cells [29] and many neurons [30]. Considering such stimulation patterns (see bottom panel of Figure 1), it appears that terminal ATP receptors would facilitate only AVP release at the beginning of such bursts. This could explain why burst durations and interburst intervals are critical for AVP release, in particular [see 8].

We, therefore, postulate that such feedback systems are important in peptide release from other, if not all, peptidergic systems. Furthermore, these mechanisms could have widespread importance, since bursting patterns of action potentials are necessary for the release of not only peptides but many other neurotransmitters in the central and peripheral nervous systems.

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Figure 1. Effect of voltage, number of bursts and frequency dependence of electrical stimulation on AVP and OT release

In electrically stimulated rat neurohypophysial glands, increasing the voltage (top panel) or the number of 25 second long bursts (3V:39Hz; middle panel) caused an increase in both AVP and OT release that peaked at approximately 3.5V and at 3 bursts. Similar effects were seen with mice (data not shown). Furthermore, increasing the frequency within an AVP like burst (four 25 second bursts separated by 21 second interburst intervals) resulted in peak AVP release at 28Hz and peak OT release at 56Hz (bottom panel).

Figure 2. Effect of suramin, a P2X2 and P2X3 receptor antagonist, on AVP and OT release
 In mouse neurohypophyseal glands, pretreatment with the selective P2X2/3 receptor antagonist suramin (5–15 μ M), decreased electrically stimulated (4 bursts; 39Hz; 3V) AVP release (panel A) indicating that endogenous release of ATP facilitates electrically stimulated AVP release. In contrast, electrically-stimulated OT release was increased in the presence of suramin (panel B) reflecting an inhibitory effect of endogenous ATP. Summary of all mouse neurohypophyseal glands pretreated with the selective P2X2/3 receptor antagonist suramin (as in panels A and B): suramin decreased electrically-stimulated AVP release by 43% ($P < .001$; $n = 16$; panel C) while increasing electrically stimulated OT by 177% ($P < .001$; $n = 15$; panel D). Comparable effects were observed in rats [7].

Figure 3. Effect of the selective P2X2 and P2X3 antagonist PPADS on AVP and OT release
 In mouse neurohypophyseal glands, pretreatment with the selective P2X2/3 receptor antagonist PPADS (5 μ M), decreased (19%) electrically stimulated (4 bursts; 39Hz; 3V) AVP release (panel A) indicating that endogenous release of ATP facilitates electrically stimulated AVP release. Electrically stimulated OT release was increased (22%) by PPADS (panel B) reflecting an inhibitory effect of endogenous ATP. Similar effects were observed in rats [7]. Furthermore, pretreatment of P2X2/3 receptor knockout mice ($n = 2$; panel C) with PPADS (5 μ M) had no effect on electrically stimulated AVP release but decreased electrically stimulated AVP release by 24% from wild type control mice ($n = 2$; panel D) similar to the effects in Panel A.

Figure 4. Effect of suramin on electrically stimulated AVP release from Wild Type, P2X3, P2X2&3 and P2X7 receptor knockout mice

In mouse neurohypophysial glands, pretreatment with the selective P2X2 and P2X3 receptor antagonist suramin (5–15 μ M), differentially decreased electrically stimulated (4 bursts; 39Hz: 3V) AVP release from wild type (panel A; 48% decrease; n=10), P2X3 receptor knockout mice (panel B: 47% decrease; n=4), P2X2&3 receptor knockout mice (panel C: 24% decrease; n=6) and P2X7 receptor knockout mice (panel D: 58% decrease; n=2).

Figure 5. Summary of effects of suramin on electrically stimulated AVP release from Wild Type, P2X3, P2X2&3 and P2X7 receptor knockout mice

Summary of the effects of suramin on electrically stimulated AVP release in all treatment groups. This data indicates that P2X2 is the primary receptor responsible for the facilitation of electrically stimulated AVP release by endogenous release of ATP. The inhibitory effect of suramin on endogenous ATP facilitation of AVP release was significantly ($p < .05$) reduced only in the P2X2&3 receptor knockout mice.

Figure 6. Effects of suramin on electrically stimulated OT release from Wild Type, P2X3, P2X2&3 and P2X7 receptor knockout mice

In mouse neurohypophysial glands, pretreatment with the selective P2X2 and P2X3 receptor antagonist suramin (5–15 μ M), increased electrically stimulated (4 bursts; 39Hz; 3V) AVP release from wild type (panel A; 94% increase; n=10), P2X3 receptor knockout mice (panel B: 174% increase; n=4), P2X2&3 receptor knockout mice (panel C: 110% increase; n=6) and P2X7 receptor knockout mice (panel D: 67% increase; n=2).

Figure 7. Model of established exogenous and proposed endogenous purinergic effects on neurohypophysial terminals

P2X2, P2X3, P2X4, & P2X7 receptors are localized on AVP terminals while only P2X7 is on OT terminals. We propose that ATP and its metabolite adenosine, have autocrine modulatory effects on the release of AVP *vs.* OT during physiological bursting patterns of stimulation. In fact, these purinergic feedback mechanisms could explain the efficacy of such patterns in facilitating AVP *vs.* OT release. We propose the following series of events; ATP that is co-released with the neuropeptides opens P2X2 and P2X3 channels, these cation channels would depolarize the RMP and, in parallel, increase intraterminal calcium concentrations. Because these receptors are exclusively on AVP terminals this would thus potentiate subsequent AVP release, in particular. Then ecto-ATPases found on the AVP terminals (Trodec, 1998) hydrolyze the extracellular ATP to adenosine. (see, also (8)).