Role of purinergic P2Y1 receptors in regulation of vasopressin and oxytocin secretion

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Song Z, Gomes DA, Stevens W. Role of purinergic P2Y1 receptors in regulation of vasopressin and oxytocin secretion. Am J Physiol Regul Integr Comp Physiol 297: R478–R484, 2009. First published June 10, 2009; doi:10.1152/ajpregu.00163.2009.—Pharmacological studies demonstrated that ATP elevates intracellular calcium ([Ca\(^{2+}\)]\(_i\)) in supraoptic nucleus (SON) neurons primarily by activation of P2X2 and P2X2/1 purinergic receptors [P2Y1R; (18)]. The current studies provide evidence for the presence of P2Y1R protein in SON neurons, evidence that activation of these P2Y1Rs induces an increase in [Ca\(^{2+}\)]\(_i\), from both intracellular stores and Ca\(^{2+}\) influx, and functional evidence that activation of P2Y1Rs induces vasopressin (VP) and oxytocin (OT) hormone release. Pretreatment of Fura-2 AM-loaded explants of the hypothalamo-neurohypophysial system (HNS) with thapsigargin (TG) significantly (~80%) reduced the increase in [Ca\(^{2+}\)]\(_i\), induced by the P2Y1R-specific agonist, 2-methylthio-ADP (2-MeSADP). In contrast, the increase in [Ca\(^{2+}\)]\(_i\) was slightly (~20%) decreased in calcium-free medium. The calcium response to 2-MeSADP was completely blocked by the P2Y1R-specific antagonist, MRS2179 or by a combination of TG pretreatment and calcium-free medium. It was absent in P2Y1 knockout mice (P2Y1R\(^{-/-}\)). 2-MeSADP significantly increased VP and OT release from perfused rat and wild-type mouse HNS explants compared with control. MRS2179 prevented this response in wild-type mouse, but it did not prevent ATP-induced hormone release from rat explants. 2-MeSADP did not induce hormone release from P2Y1R\(^{-/-}\) explants. These findings support a potential role for P2Y1Rs in regulation of VP and OT release. The finding that P2Y1R activation induces a small Ca\(^{2+}\) influx suggests that P2Y1Rs may regulate VP release by modifying ion channels such as stretch-inactivated cation channels.

ATP; hypothalamus; neurohypophysial; supraoptic nucleus

ATP is an important neurotransmitter in the pathway carrying information about hypovolemia and hypotension to the vasopressin neurons in the supraoptic nucleus (SON). Previous studies have identified this pathway as the catecholaminergic projection from the A1 neurons in the ventrolateral medulla, and have shown that ATP is a cotransmitter in the pathway (4, 5). ATP activates purinergic receptors that include both a family of ligand-gated ion channels, the P2X receptors (P2Xs, 1-7), and a family of G protein-coupled receptors, the P2Y receptors [P2YRs, 1-14 (14, 22)]. SON neurons express mRNA for several P2XRs subtypes (16), and activation of either P2XRs or P2YRs increases intracellular calcium ([Ca\(^{2+}\)]\(_i\)) in SON neurons. ATP induces both calcium influx and release of calcium from intracellular stores in SON neurons (18). The former reflects activation of P2XRs and voltage-gated calcium channels, while the latter reflects activation of P2YRs. The P2Y1R subtype is predominantly responsible for ATP-induced release of calcium from intracellular stores, as indicated by the ability of a P2Y1R specific antagonist, MRS2179, to eliminate the ATP-induced increase in [Ca\(^{2+}\)]\(_i\) in the absence of extracellular Ca\(^{2+}\) (18). Furthermore, the P2Y1R agonist, 2-methylthio-ADP (2-MeSADP) induced large increases in [Ca\(^{2+}\)]\(_i\) in all SON neurons tested, while UTP, an agonist at P2Y2 and 4Rs, or UDP, an agonist for P2Y6Rs subtypes, only induced small increases in [Ca\(^{2+}\)]\(_i\) in a minority of SON neurons (18).

The current studies provide molecular evidence for expression of P2Y1R in SON and paraventricular nucleus (PVN) neurons, as well as evidence that P2Y1R activation stimulates vasopressin (VP) and oxytocin (OT) release from the posterior pituitary. The source of the P2Y1R-induced increase in [Ca\(^{2+}\)]\(_i\) is characterized as well.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats [CRL:CD(SD)Br; Charles River Laboratories, Wilmington, MA], male C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME), and male P2Y1R\(^{-/-}\) mice (Taconic Farms, Hudson, NY) were used. The P2Y1R\(^{-/-}\) mouse colony was developed and characterized by Fabre et al., UNC (8). All protocols used were performed in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of the University of Colorado Denver.

Western Blot Analysis

SON was microdissected from male rats (125–150 g) or mice (15–25 g) and homogenized in cold buffer [tetrasodium pyrophosphate (20 mM), sodium phosphate (20 mM), MgCl\(_2\) (0.5 mM), sucrose (300 mM), benzamidine (0.8 mM), iodoacetamide (1.0 mM), leupeptin (1.1 mM), pepstatin A (0.7 μM), PMSF (0.23 mM), and aprotonin (76.8 μM)] using a dounce homogenizer. The homogenate was centrifuged at 42,000 rpm for 1 h, and the resulting ATP was loaded onto a 10% SDS-PAGE gel and transferred to nitrocellulose. The P2Y1R antibody was used at a 1:1,000 dilution and detected with an enhanced chemiluminescence system. A Western blot of homogenates from rat and wild-type mice is shown in Fig. 1.

Fig. 1. Immunoblot of microdissected supraoptic nucleus (SON) homogenates (40 μg protein per lane) from rat and wild-type (WT) or P2Y1R\(^{-/-}\) mouse. A band at 42 kDa was revealed in rat SON. Another band at ~60 kDa was revealed in rat and WT mouse, but not in P2Y1R\(^{-/-}\) mouse. Preabsorption of the antibody with the immunogenic peptide eliminated both bands (Ab\(^{-}\)).

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pellet was solubilized in Laemmli buffer with beta mercaptoethanol and denaturing protein complexes by heating at 85°C for 3 min. Protein concentration of the extracts was determined using Biosciences CB-X Protein Assay Kits. Extracts were subjected to SDS-PAGE, transferred to nitrocellulose, blocked with 5% nonfat milk, and incubated with primary antibody (P2Y1R, Sigma, 1:200) in 5% nonfat milk PBS overnight at room temperature. The nitrocellulose blot was washed 3 times for 10 min. in 0.1% Tween followed by 3 PBS rinses and incubated with peroxidase-conjugated donkey anti-rabbit IgG (1:5,000) for 2 h at room temperature. Immunoreactivity was detected using the Pierce Supersignal West Femto Kit. Preabsorption of the antibody with the peptide immunogen eliminated reactive bands.

Immunohistochemistry

Male rats (250–300 g) or mice (15–25 g) were anesthetized with Avertin (1 ml/100 g body wt delivered IP), and the brain was perfused transcardially with phosphate-buffered 4% paraformaldehyde, post-fixed in 4% paraformaldehyde for 2–3 h, allowed to sink in 30% aqueous sucrose for 48 h, and cryostat sectioned at 30 μm. Sections
were stored in cryoprotectant solution at −20°C until processed for staining. All rinses and incubations, except the antibody incubation, were performed at room temperature (RT). Free-floating sections were rinsed in 0.05 M KPBS for 1 h and then blocked for 1 h in 1% normal goat serum and 5% BSA, followed by 48-h incubation at 4°C in an affinity-purified rabbit polyclonal P2Y1R antibody from Sigma (epitope = aa 242-258 of P2Y1R) diluted 1:500 in KPBS/0.4% Triton X. The sections were rinsed for 1 h in KPBS and incubated for 1 h in biotinylated anti-rabbit IgG in KPBS/0.4% Triton X. After 30-min rinse in KPBS, sections were incubated in ABC reagents (Vector ABC Elite Kit) for 1 h. Finally, tissue sections were treated with the chromogen solution consisting of diaminobenzidine, H2O2, and NiSO4. The sections were rinsed in KPBS and mounted onto polylysine-treated slides. Air-dried slides were dehydrated in ethanol, cleared in xylene, and cover-slipped. Staining specificity was determined by the absence of staining following preincubation of the primary antibody with the unconjugated peptide antigen at a ratio of 0.5 μg peptide: 1 μg antibody for 3–4 h at 4°C (Fig. 2B), and the absence of staining in hypothalamic sections from P2Y1R knockout mice (Fig. 2D).

**Hypothalamo-Neurohypophyseal Explant Preparation**

Explants of the hypothalamo-neurohypophyseal system (HNS) were used for calcium imaging and hormone release studies. Explants were prepared as described previously from male rats (125–150 g) or mice (15–25 g) (19). HNS explants include the SON neurons, their axons, and axon terminals in the neural lobe, as well as organum vasculosum of the lamina terminalis, and suprachiasmatic and arcuate nuclei. They do not include the PVN.

**Calcium Imaging**

HNS explants were loaded with the calcium-sensitive dye, Fura-2 AM, as described previously (19). They were placed in a recording chamber with the ventral surface up allowing easy visualization of SON neurons using the optic chiasm as an anatomical landmark (19). Magnocellular SON neurons were identified by the size of the cell body (>25 μm in diameter) and their location adjacent to the optic chiasm. Explants were perfused at a rate of 3 ml/min with gassed (95% O2-5% CO2) F12 nutrient mixture fortified with 1 mg/ml glucose and 1.7 mM CaCl2, and Fura-2-loaded magnocellular neurons were alternately excited with 340 nm and 380 nm UV light from a Xenon Source (Sutter Instruments, Novato, CA). The 380-nm exposure time was between 200 and 500 ms and was tripled for the 340-nm exposure. Emitted light was passed through a ×60 fluor water immersion lens attached to an Olympus upright microscope and collected at 510 nm by an intensified charge-coupled device camera (Hamamatsu, Tokyo, Japan). Paired 340- and 380-nm images were acquired every 3 s using Slide-Book software (Intelligent Imaging, Denver, CO) for a period of 100 frames. The 340:380 ratio (R) was used as an index of the change in [Ca2+]i. An example image of the ratio and 340/380 recordings from representative SON neurons has been shown previously (19). R max was determined in ionomycin-AJP-Regul Integr Comp Physiol • VOL 297 • AUGUST 2009 • www.ajpregu.org
treated explants and far exceeded the highest R obtained with agents studied in these experiments (19). R data are presented as a percentage of the basal 340:380 R for each cell determined from the average R of 10 frames preceding drug exposure. Explants were allowed to equilibrate for 1 h. Mean ± SE of the percentage values from individual neurons were calculated and plotted. Parametric one-way ANOVA (F value) followed by Student-Newman-Keuls individual mean analysis or Kruskal-Wallis one-way ANOVA on ranks (H value) followed by Dunn’s individual mean analysis was used to determine significant group differences in the peak responses.

Hormone Release From HNS Explants

Explants were positioned individually in perfusion chambers having a 500-μl volume, and perfused with modified F12 nutrient mixture at 2 ml/h, as described previously (17). Following a 4- or 5-h equilibration period to allow hormone release to stabilize at basal level, explants were either maintained under control conditions or exposed to purinergic agents as appropriate for each experiment. Effluent was collected individually at 20-min intervals using a refrigerated fraction collector maintained at 4°C. VP and OT concentration in the perfusate was determined by radioimmunoassay, as described previously (23). VP and OT release from HNS explants reflects changes in hormone release from nerve terminals in the neural lobe, because although VP and OT are released from dendrites in SON and VP from suprachiasmatic nucleus (7), the amount from neural lobe far exceeds these other sources (9).

Data analysis. Basal VP/OT release was determined during the hour immediately preceding exposure to drugs. Hormone release in response to experimental manipulations is expressed as a % of this initial basal release for each explant. Basal release for the explants included in these studies was 90.63 ± 29.4 pg/ml for VP and 127.61 ± 21.75 pg/ml for OT (mean ± SE). ANOVA with repeated measures followed by post hoc simple main effects analysis was performed to evaluate changes in hormone release and to compare responses between groups.

RESULTS

P2Y1R expression in SON

The presence of P2Y1R protein in SON and PVN was demonstrated with immunohistochemistry and Western blot analysis. Immunoblotting with an antibody against P2Y1R from Sigma (1:200 dilution) revealed a band at 42 kDa in the rat, which was consistent with the predicted size of P2Y1R. Another larger band (~60 kDa) was detected in the rat and WT mouse, but was absent in P2Y1R-/− mouse. Its absence in P2Y1R−/− mice suggests that it is a post-translationally modified form of the receptor protein, which is predominant in mice. Neither band was detected when the antibody was preabsorbed with immunogenic peptide (Fig. 1). As shown in Fig. 2A, rat SON neurons were intensely labeled following incubation with the same P2Y1R antibody. Both magnocellular and parvocellular neurons of rat PVN were immunoreactive for P2Y1Rs (Fig. 2E). Accessory magnocellular neurons in nucleus circularis (Fig. 2F) and retrochiasmatic SON (not shown) were also immunoreactive. Immunoreactivity was absent in rat SON following preabsorption of the antibody with the peptide antigen (Fig. 2B, Ab−). P2Y1R-immunoreactive neurons were also present in mouse hypothalamus, including SON (Fig. 2C) and PVN (not shown). Antibody specificity was demonstrated by the absence of immunoreactivity in hypothalamic sections from P2Y1R knockout (P2Y1R−/−) mice (Fig. 2D).

P2Y1R Activation Induces Ca2+ Release From Intracellular Stores

We previously reported that the P2Y1R specific agonist, 2-MeSADP, induced large increases in [Ca2+], that were comparable in the presence and absence of tetrodotoxin (18). Figures 3 and 4 present further evidence that this reflects activation of P2Y1Rs. The 2-MeSADP-induced increase in [Ca2+]i was prevented by the P2Y1R specific antagonist, MRS2179 (Fig. 3, C and D; H = 44.262, P < 0.001). Furthermore, in P2Y1R−/− mouse explants, ATP was able to induce an increase in [Ca2+]i, but subsequent exposure of the same explants to 2-MeSADP did not change [Ca2+]i, (Fig. 4). Example 340, 380 recordings and the calculated ratio (340/380) from representative SON neurons treated with 2-MeSADP are also shown (Fig. 3, A and B).

As expected for activation of a Gq/11 linked receptor, the increase in [Ca2+]i, induced by 2-MeSADP primarily reflects calcium release from intracellular stores, because pretreatment with 200 nM thapsigargin (TG) for 30 min to deplete intracellular calcium stores eliminated ~80% of the 2-MeSADP-induced increase in [Ca2+]i. However, ~20% of the response reflects extracellular Ca2+ influx, because the response was significantly reduced during incubation in zero external calcium. Combined pretreatment with TG and incubation in zero external calcium was required to completely block the response (H = 139.01, P = <0.001. Fig. 5, A and B). This is important, because it demonstrates that P2Y1Rs have potential to modify Ca2+ influx through other ion channels.
**P2Y1R Activation Induces VP and OT Release**

2-MeSADP, the P2Y1R-specific agonist, induced significant increases in both VP and OT release from perifused rat HNS explants compared with explants perifused with basal medium (Fig. 6A, VP: \( F_{\text{time}} = 4.848, P < 0.001, F_{\text{group}} = 1.389, P = 0.257 \); and Fig. 6B, OT: \( F_{\text{time}} = 3.766, P = 0.003, F_{\text{group}} = 0.193, P = 0.668 \). This suggests that activation of P2XRs and/or other P2YR subtypes primarily accounts for mediated hormone release by ATP alone.

**DISCUSSION**

In its role as a neurotransmitter, ATP activates both fast-acting ionotropic receptors and metabotropic (G protein-coupled) receptors that signal via activation of slower enzymatic processes. As was shown previously (18) and is further substantiated with the data presented here, G protein-coupled, purinergic receptors of the P2Y family contribute to ATP-induced increases in \([\text{Ca}^{2+}]_i\) in SON neurons and stimulate VP and OT release from the neurohypophysis. Because metabotropic receptors are potentially important for regulating components of an integrated response, such as regulation of receptor trafficking and induction of gene expression to maintain hormone secretion, it is important to fully characterize the contribution of the metabotropic P2YRs to ATP-mediated responses.
The robust and uniform expression of P2Y1R immunoreactivity throughout the dorsal/ventral extent of SON attests to the expression of P2Y1Rs in both VP and OT neurons. This is consistent with the finding that all imaged SON neurons responded to the P2Y1R agonist, 2-MeSADP, as well as the stimulation of both VP and OT release by 2-MeSADP. The expression of P2Y1R in both magnocellular and parvicellular PVN neurons suggests that ATP may alter autonomic, as well as neuroendocrine functions of PVN.

In contrast to the immediate and comparable increase in [Ca\(^{2+}\)], induced by both ATP and 2-MeSADP, the hormone release response induced by these two agonists is different: 1) The time course of the hormone response is different. The response to ATP is rapid, but transient (11), while the response to 2-MeSADP is slower and sustained, with the peak increase in VP and OT release induced by 2-MeSADP occurring 1 h after the addition of the drug, and hormone release remaining elevated relative to control explants throughout 3 h of exposure to 2-MeSADP (Fig. 6). 2) Even though activation of P2Y1Rs contributes about 50% to ATP induced [Ca\(^{2+}\)] increase (18), it is not required for ATP stimulated hormone release (Fig. 8). However, it may be necessary in some other ATP-mediated physiological processes, such as ATP-stimulated synergistic hormone increase in the presence of norepinephrine. The difference in the hormone release response to ATP and 2-MeSADP is consistent with the ionotropic P2XRs and the G protein-coupled P2Y1Rs playing different roles in regulation of hormone release and probably reflects different cellular responses activated by P2X and P2Y1Rs. ATP depolarization of SON neurons through activation of P2XRs (10) can be expected to trigger action potentials which, in turn, initiate exocytosis at the nerve terminals and thus induce immediate hormone release from the neural lobe. In contrast, evidence for P2Y1R-mediated depolarization of SON neurons is scant. Although 2-MeSATP mimicked the depolarizing effect of ATP on SON neurons (10), 2-MeSATP is a potent agonist at P2X3Rs (14), as well as P2Y1Rs. Therefore, the observed depolarization could reflect activation of P2X3Rs rather than P2Y1Rs, and P2X3R mRNA is present in SON neurons (16). ADP, a more selective P2Y1R agonist (22), induced only a small depolarization compared with ATP (10). On the other hand, activation of P2Y1Rs could produce inositol triphosphate and 1, 2-diacylglycerol by activating PLC, thus releases Ca\(^{2+}\) from intracellular stores and may activate Ca\(^{2+}\)-dependent PKC. This could lead to new gene expression or altered receptor trafficking as a result of PKC-mediated phosphorylation events. The delayed increase in hormone release in response to 2-MeSADP is...
consistent with activation of these slower cellular events that eventually lead to an increase in hormone release.

Perspectives and Significance

Osmotic transmission in SON is subject to modulation by other physiological parameters. Excitatory peptides, such as ANG II, CCK, and neurotensin, can excite HNS via modulation of the osmosensitive stretch activated cation (SIC) channels (2, 3). Blood volume loss results in greater increase in osmotically stimulated plasma VP. This suggests modulation of osmoreception by neurotransmitters conveying hemodynamic information (6, 20). Both P2Y1 and 2Rs have been shown to activate or modulate TRPV1 channel activity, possibly through the activation of PKC (1, 12, 15, 21). A recent report revealed that the SIC channel, which functions as the osmosensor in SON is a N-terminal variant of the TRPV1 channel (13). Thus, P2Y1 and/or 2Rs could potentially modulate SIC channel activity via PKC in SON. Our current finding that activation of P2Y1Rs by 2-MeSADP induced a small component of Ca2+ influx in addition to stored release of Ca2+ is in agreement with this postulation. It is possible that ATP, a neurotransmitter in the A1 pathway carrying cardiovascular information to SON (4, 5), can augment SIC channel activity via activation of P2Y1 and/or 2Rs. This could be the cellular mechanism underlying the well-described modulation of osmotic regulation of plasma VP by hypovolemia and hypervolemia. Thus, additional studies are required to verify P2YR modulation of SIC channels, it is evident that P2Y1Rs in SON can play an important role in regulation of neurohypophyseal hormone release.

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