ATP modulates Hypothalamic Oxytocin Exocytosis via Mitochondrial Calcium Release

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Abstract:
Background: The use of 56mM potassium chloride (KCl) to stimulate neurotransmitter or hormone release from central neurons is canonical but has been criticized for being nonphysiological. Recent data from this laboratory suggest that Adenosine triphosphate (ATP) is involved in a nonmetabolic way acting as a direct or indirect stimulant of oxytocin release from spinal cord synaptosomes.
Materials and Methods: We have examined the possibility that ATP has an effect in modulating the release of oxytocin from hypothalamic synaptosomes. To test this hypothesis, we used hypothalamic synaptosomes obtained from 30 day old Sprague-Dawley rats.
Results: Data obtained from these experiments demonstrated that ATP caused a 200 % increase of oxytocin release over control. ATP-induced release was not inhibited by pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid (PPADS), a P2X 1,2 or 3 purinergic receptor antagonist. Results obtained with cholecystokinin (CCK-8) also showed a stimulatory effect of oxytocin release from hypothalamic synaptosomes. CCK 8- induced oxytocin release was also not inhibited by LY225910, a well known CCK-8 receptor antagonist. Release of oxytocin by CCK-8 and ATP was achieved in calcium free media. ATP-induced release of oxytocin was inhibited by prior application of GCP37157, a mitochondrial calcium efflux channel antagonist indicating mitochondria as the source of calcium.
Conclusion: These data suggest that calcium sequestration and release by mitochondria may play an important role in the control of ATP mediated oxytocin release from hypothalamic synaptosomes.
Key Words: Mitochondria, ATP, Oxytocin, Calcium, Exocytosis

Introduction

Magnocellular neurons of the hypothalamus found in the supraoptic nucleus and the paraventricular nucleus are the primary producers of Oxytocin (OT). The majority of this secretion travels via axons of the infundibulum to the posterior pituitary where it is released into the pituitary portal system as a hormone [1].

OT and OT receptors (OTRs) are found in many locations in the central nervous system. Recently, OT release has been identified in visceral organs such as the stomach, small intestine, kidney and heart (unpublished data of Robert Parks and Jaya Haldar 2010). The extensive anatomical presence of OT and OTRs indicates that OT might play a significant role in many physiological systems.

The classic hormonal activities of OT, eg. parturition, and milk ejection , are well known. But, OT mediates higher order behavioral activities. Maternal behavior is closely associated with OT release in cortical synapses. Complex social interactions, such as monogamy have been related

to OT and OTR activity [2]. Interactions between OT and digestive hormones, such as gherlin and leptin indicate a linkage with feeding behaviors such as satiety or the initiation of feeding [3].

Previous results from our laboratory suggests that ATP and the mitochondria may be important in the regulatory mechanism that controls the release of OT as a neurotransmitter in the spinal cord of rats (Chowdhury,N. Ph.D thesis 2004). Rotenone (RT) and potassium cyanide (KCN), both classical inhibitors of the electron transport chain, strongly inhibited OT release from spinal cord synaptosomes. These observations suggested that ATP has some role in OT release.

The current study was undertaken in an attempt to understand the release mechanism of OT from central neurons. Specifically, to clarify the role of ATP in this system.

MATERIALS AND METHODS

Dissection

For each experiment, three one month old, 75-100 gram Sprague-Dawley male rats were sacrificed by decapitation. The brains were quickly removed from the skull, excess blood washed from the brain with ice cold phosphate buffered saline (PBS) . Four incisions were made in the brain: i) anteriorly to remove the frontal cortex up to the optic chiasm, ii) posteriorly to remove the brainstem and cerebellum, iii) laterally on right and left to remove the temporal cortex, and iv) superiorly, to remove the parietal cortex. A block of tissue remained which contained the following: i) the optic chiasm, anteriorly, ii) the posterior end of the third ventricle, iii) the medial eminence on the inferior border, and iv) superior end of the block contains the superior portion of the third ventricle. The resulting dissected tissue contained the hypothalamic neurons of interest, namely the paraventricular and supraoptic nuclei that contain large numbers of oxytocinergic neurons (Figure 1). The tissue from all 3 animals were pooled, weighed and sucrose gradient buffer (SGB) as a volume at 5 times the weight of the pooled tissue was added. The composition of SGB is 320mM sucrose, 1mM EDTA and 0.25mM dithieothreitol (DTT). The tissue was homogenized with 8-10 strokes of a Dounce homogenizer. Sample

Synaptosome preparation by differential centrifugation

All centrifugations were performed at 4^0 C. The homogenized tissue was centrifuged at 3600 x g for 10 minutes. The supernatant fluid was collected into a cold centrifuge tube. The pellet was resuspended in 2 ml of SGB and centrifuged again for 10 minutes at 3600 x g. This supernatant was added to the supernatant fluid. The combined supernatant fluid was carefully layered onto a column of sucrose gradient consisting of 3%, 12%, 15%, and 23% sucrose from top to bottom. The sample was centrifuged at 11,000 x g for 11 minutes . The purest synaptosomal fraction was found in a band between the 15% and 23% sucrose. This band was collected with a Pasteur pipette and washed twice with cold Krebs buffer and collected by centrifugation at 1500 x g [4,5,6] and visualized by transmission electron microscopy (Figure 2).

Incubation

After washing, the entire yield of synaptosomes were resuspended in 3.3ml of incubation media (1540 mM NaCl, 56 mM KCl, 10 mM $MgCl_2$, 0.25% BSA, 100 mM glucose, 100 mM HEPES), the suspension is mixed on vortex and divided into equal portions of 500 ul. In all experiments samples were incubated for 3 minutes. The remaining volume was saved for determination of protein content [7]. Antagonists were always added 1 min. prior to the stimulant, except for the mitochondrial Ca^{++} channel blocker CGP 37157, that was added three minutes prior to stimulation [8,9].

Quantification of OT release by Radioimmunoassay (RIA)

The OT molecule contain one disulfide bridge between the Cys in positions 1 and 6. RIA uses the Tyr in position 2 for attachment of an I^{125} label, which is essential for quantification of OT. The low molecular weight (1007) of OT makes its detection difficult by electrophoretic methods. Thus, the use of RIA becomes important, due to the fact that this assay has very high sensitivity. The RIA can detect quantities as low as 1 pg of OT. At the end of three minute incubation period, tubes were spun in a microcentrifuge for one minute and

the supernatant fluid was collected from each sample. The sample from each incubation experiment was assayed using RIA. The unknowns were incubated with anti-OT antibody for 5 days followed by incubation with I^{125} OT for 2 days. The unknowns were compared to a standard curve and the quantities of OT released from each experiment was expressed in picograms (pg). After Biorad protein determination for each experiment, the pg OT release was standardized to pg/mg protein for preparation of charts.

Materials

Percoll for sucrose gradient purchased from Fischer Bioscience.

Antibody used in RIA was a polyclonal anti-OT antibody created and donated by Dr. Robert Feldman.

Hypothalamic synaptosomes were prepared from one month-old, 75-100 gram Sprague-Dawley male rats. Three animals per experiment were obtained from Taconic labs, Elmont, N.Y. The animals were quickly sacrificed by cervical dislocation and decapitation. This was done as quickly as possible and every effort was made to minimize suffering or stress to the animals. Protocols for animal care and use were strictly adhered to as prescribed by the committee on Animal care and Use of St. John's University, Queens, N.Y. All animal protocols were done with strict adherence to the Guide.

RESULTS

Cholecystokinin (CCK8) has been shown to be a good physiologic stimulant of OT release from synaptosomes derived from spinal cord and other brain areas (Chowdhury & Haldar, unpublished data). To determine whether CCK 8 is an effective stimulant for hypothalamic synaptosomes as well, we used different concentrations of CCK8. Dose response curve obtained with CCK8 demonstrates that 50nM CCK8 gave the strongest and most consistent release of OT from the hypothalamic synaptosomes. Therefore, in all following experiments 50nM CCK-8 has been used as the positive control . These data in figure 3 represent an average of five experiments. (see Altimari,et.al. 2015)_

Similar to CCK8 experiments, ATP also causes OT release from hypothalamic synaptosomes The 50nM dose of ATP strongly stimulated OT release from hypothalamic synaptosomes. These data represent an average of four experiments.

It is known that calcium is required for exocytosis of neurotransmitter or hormone in most systems [10,11]. However, in our experiments, both with CCK8 and ATP, the incubation media used was calcium (Ca^{++}) free. This raises the obvious question ,what is the source of Ca^{++} for OT release in this system? Previous work from our laboratory indicated that mitochondria might be involved in the release mechanism (Chowdhury, N, thesis 2005). To determine whether mitochondria indeed was the source of intracellular Ca^{++}, we conducted some experiments with CGP 37157, a specific mitochondrial calcium channel blocker. Data presented in figure 4 show that after 3 minutes of incubation with CGP 37157 followed by 50nM ATP there was strong inhibition of OT release from hypothalamic synaptosomes. These experiments demonstrate that the source of calcium that is required for the release of OT is most probably the mitochondria. The ATP dose response chart in figure 3shows strong release of OT in calcium free media in response to 50 nM ATP.

DISCUSSION

CGP 37157 is a specific mitochondrial calcium efflux channel blocker. It is a sodium/ calcium exchanger. ATP could be stimulating these channels by depolarization of voltage gates with a resultant efflux of calcium which would stimulate the exocytosis machinery with the ultimate release of OT. These data suggest that CGP 37157 blocks the exchanger and causes calcium to be sequestered in the mitochondrial matrix and thus strongly inhibiting release of OT from the synaptosomes. The results with CGP 37157 point to the mitochondrion as the organelle that is regulating calcium concentrations during the exocytosis of OT.

Since synaptosome preparation is an *in vitro* technique it excludes many variables that maybe important in living cells including most plasma membrane and ER components. But synaptosomes allow for a more controlled analysis of one aspect of a problem. The differential centrifugation in the preparation steps results in a suspension of synaptic vesicles in various states of priming and a few mitochondria.

In conclusion, the data in these studies can be used to support a simple model of cellular regulation of OT release. This model includes depolarization of mitochondrial calcium voltage gated channels with the resultant stimulation of vesicles via the SNARE pathway [15].

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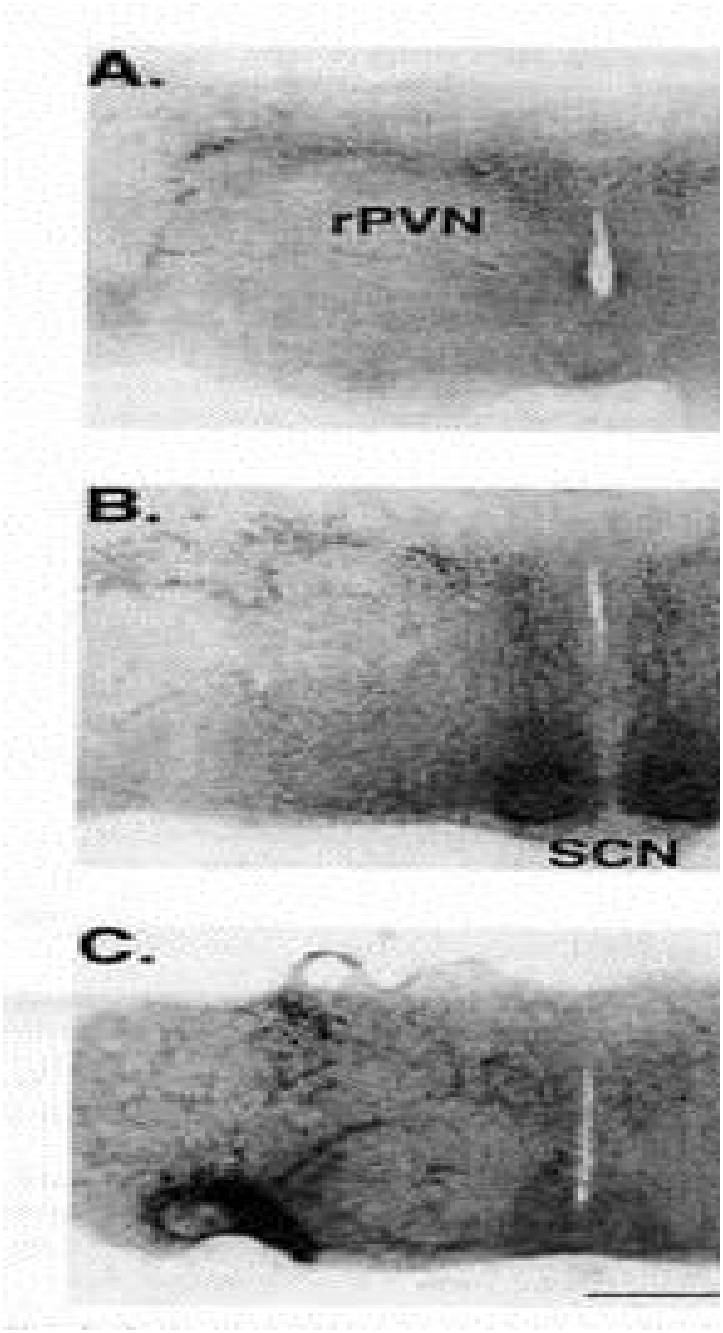

Fig. 1. Gross Anatomy of the Hypothalamus The sections are from Anterior to posterior. Section A is most rostral(Anterior) and Section C is most Posterior. The paraventricular nucleus (rPVN) and the supraoptic nucleus (SON) can be seen in their classical relationship to the third ventricle. These two nuclear regions are the primary locations for oxytocin synthesis in the hypothalamus. This location was used to prepare all synaptosomes.

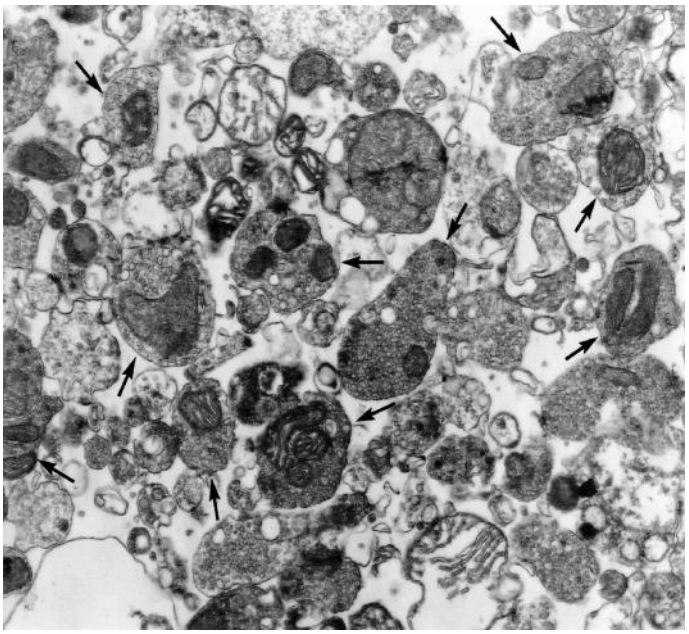

Fig. 2. Transmission electron micrograph of synaptosome preparation. In this TEM the synaptosomes can be seen (at arrows in figure). A fragment of a mitochondrian can be seen in lower right of this figure.

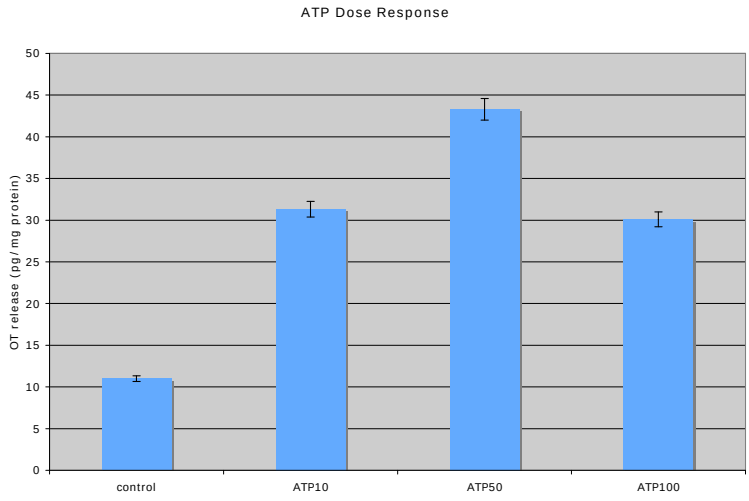

Fig. 3: ATP dose response curve. ATP dose is in nM. Error bars are 1% SEM. (average of 5 experiments each) ATP is a strong stimulant of OT from hypothalamic synaptosomes. 50nM dose appears to be best. The dose response follows the endocrine type of stimulus /release where there is an optimal stimulus concentration (50nM ATP) which builds and then drops off (at 10nM and 100nM ATP).

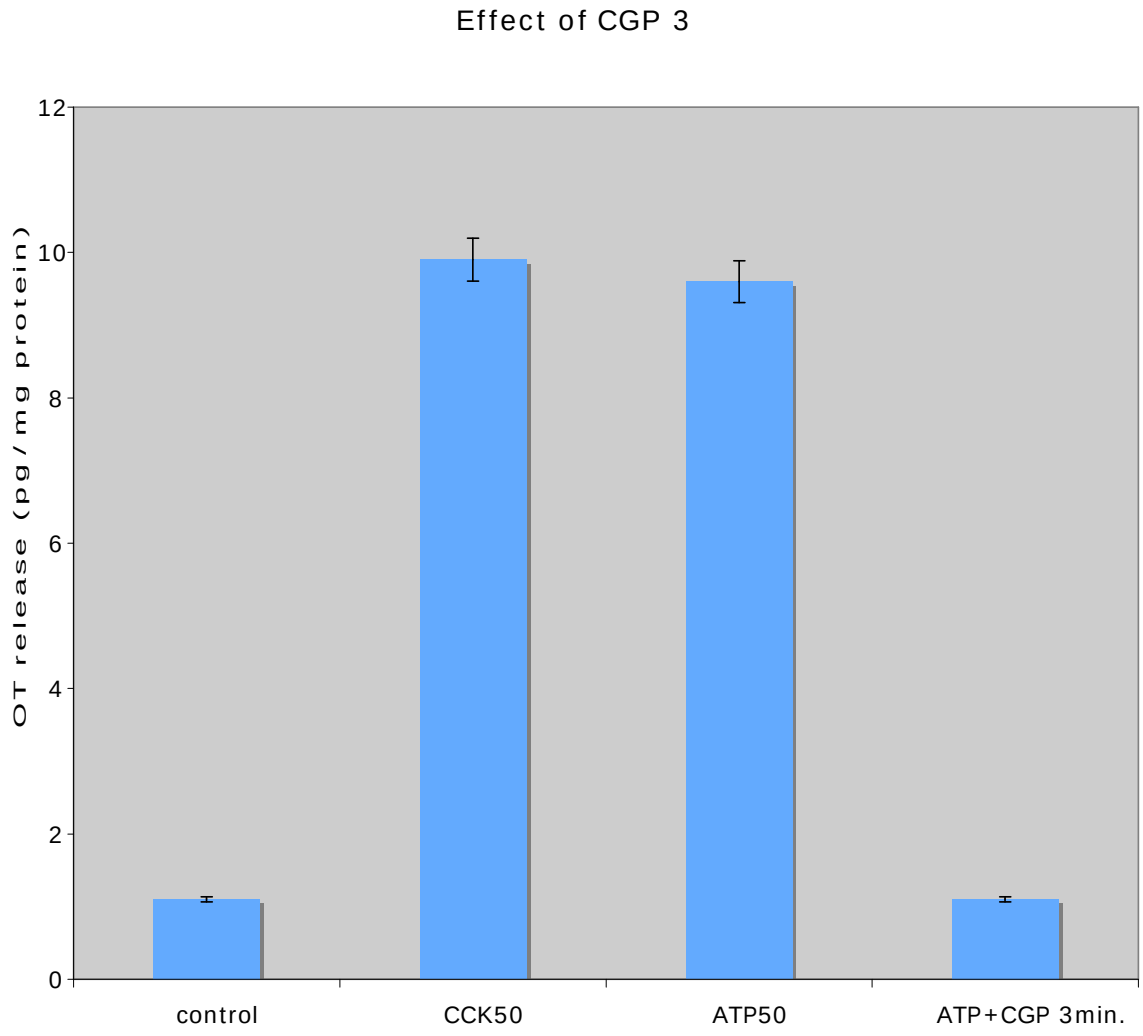

Fig. 4: The effect of CGP 37157

CGP 37157 is a specific mitochondrial Ca^{++} efflux inhibitor. ATP mediated release of OT is clearly inhibited by CGP 37157. Error bars are 1% SEM. (average of 5 experiments each) These data demonstrate that the calcium needed for the release of OT in hypothalamic synaptosomes is coming from the mitochondria.