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Effects of calcium and sodium on ATP-induced vasopressin release from rat isolated neurohypophysial terminals

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Abstract

ATP-receptors (P2X2, P2X3, P2X4 & P2X7) are found in neurohypophysial terminals (NHT). These purinergic receptor subtypes are known to be cation selective. Here we confirm that both sodium (Na⁺) and calcium (Ca²⁺) are permeable through these NHT purinergic receptors, but to varying degrees (91% vs. 9%, respectively). Furthermore, extracellular calcium inhibits the ATP-current magnitude. Thus, the objective of this study was to determine the effects of extracellular Na⁺ vs. Ca²⁺ on ATP-induced vasopressin (AVP) release from populations of rat isolated NHT.

ATP (200 μ M) perfused exogenously for 2 minutes in Normal Locke's buffer caused an initial transient increase in AVP release followed by a sustained increase in AVP release which lasted for the duration of the ATP exposure. Replacing extracellular NaCl with NMDG-Cl had no apparent effect on the ATP-induced transient increase in AVP release but abolished the sustained AVP release induced by ATP. Furthermore, removal of extracellular calcium resulted in no ATP-induced transient increase in AVP release, but had no effect on the delayed, sustained increase in AVP release.

The ATP-induced calcium-dependent transient increase in AVP release was >95% inhibited by 10 μ M of the P2X purinergic receptor antagonist PPADS, a dose sufficient to block P2X2 and P2X3 receptors but not P2X4 or P2X7 receptors. Interestingly, the ATP-induced calcium-independent, sodium-dependent sustained increase in AVP release was not affected by 10 μ M PPADS. The ATP-induced calcium-dependent transient increase in AVP release was not affected by the P2X7 receptor antagonist BBG (100 nM). However, the ATP-induced sodium-dependent sustained AVP release was inhibited by 50%.

Therefore, these results show that rat isolated NHT exhibit a biphasic response to exogenous ATP that is differentially dependent on extracellular calcium and sodium. Furthermore, the initial transient release appears to be through P2X2 and/or P2X3 receptors and the sustained release is through a P2X7 receptor.

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INTRODUCTION

Extracellular adenosine tri-phosphate (ATP) is a purine known to exert effects on a variety of tissues through specific purinergic (P1 and P2) receptors. ATP was first shown to affect sensory neurons (15) and further experiments have revealed that extracellular ATP modulates a family of channels (P2X1–P2X7) that are permeable to Na⁺, K⁺, and Ca²⁺ (1). Furthermore, all of these receptors appear to play a role in regulating the release of neurotransmitters (10). Consequently, ATP can enhance both long-term potentiation and long-term depression, but may differentially affect synaptic plasticity depending upon which type of receptor it activates and whether that receptor is located pre- or post-synaptically (9, 10, 23, 29). Thus, ATP receptor localization at CNS synapses is critical, but, for the most part, unknown.

ATP has been shown to activate a ligand-gated ion channel in cholinergic nerve terminals (26). Furthermore, in neurohypophysial terminals, exogenously applied ATP has been shown to directly increase intracellular calcium which presumably is responsible for the observed release of vasopressin and to a lesser degree oxytocin (28). Vasopressin is a vasoconstrictor and an antidiuretic (see 16 for review) which has also been implicated in social behaviors (11), stress, learning, and memory processes (7), as well as the development and maintenance of tolerance to ethanol (24).

We have previously shown that vasopressin-containing NHT have P2X2, P2X3, P2X4, and P2X7 receptors and that the majority of the ATP-induced current is due to activation of the P2X2 and P2X3 receptors, but that only P2X7 receptors colocalize to oxytocin-containing terminals (12). These purinergic receptor types are all known to be cation (*e.g.*, Na⁺ and Ca²⁺) selective (22).

The objective of this study was to determine the effects of extracellular calcium and/or sodium on ATP-induced AVP release from a population of rat isolated neurohypophysial terminals and to determine if different types of purinergic receptors might be responsible for the biphasic nature of ATP-induced AVP release. Since there are different types of purinergic receptor on AVP-containing terminals, what are their functional effects on release? Do P2X2 and/or P2X3 receptors potentiate AVP release through the influx of calcium or sodium through their channels? In view of the fact that Na⁺ can cause release independently of Ca²⁺ (25), what about intraterminal mechanisms for each receptor? Importantly, because extracellular Ca²⁺ is depleted during physiological activity (20), such differences in dose, kinetics, and intracellular mechanisms could play an important role during bursting stimulation of neuropeptide release from these NHT.

METHODS

Isolation of nerve endings

Male Sprague-Dawley CD rats (Taconic Farms, Germantown, NY) weighing 200–250 g were immobilized and immediately decapitated (2). Briefly, the animals were killed by decapitation with a guillotine following the guidelines laid down by the UMMS ethical committee and their pituitaries isolated. Following removal of the anterior and intermediate lobes of the pituitary, the neural lobe was dissociated in a buffer at 37°C that contained (in mM): sucrose, 270; EGTA, 1; HEPES-Tris, 10, buffered at pH 7.3. The dissociated neural lobe was centrifuged at $100 \times g$ for 1 minute, with the supernatant spun at $2400 \times g$ for 3 minutes. The resulting pellet, containing highly purified NHT, was resuspended in Normal Locke's (NL) saline at 37°C and containing (in mM): NaCl 140; KCl 5; CaCl_2 2.2; HEPES 10; Glucose 10; MgCl_2 1; pH 7.3, and the osmolarity was 295–300 mOsm/L. The terminals were then equally loaded onto filters (Acrodisc, LC13, 0.45 μm , Gelman Scientific, Ann Arbor, MI).

Electrophysiology

Dissociated isolated NHT, from male Sprague-Dawley CD rats (Taconic Farms, Germantown, NY) weighing 200–250 g, were obtained similarly as for release studies (see 12,13,14, 18). The ATP receptor mediated inward cation currents were obtained using the perforated-patch whole-cell recording technique (13) utilizing 2–4 M Ω resistance pipettes. Perforation of the terminals' membranes was obtained by adding 30 μM amphotericin B to the pipette solution (130 mM CsGlutamate, 10 mM HEPES, 20 mM Tetraethylammonium Chloride, 1 mM MgCl_2 , pH 7.2, at ~ 295 mOsm.). After perforation the terminals were voltage-clamped at -80 mV and perfused continually with the control NL. Selected agonists and antagonists in ATP experiments were applied for 2 seconds (solution switch controlled by an SF-77B Perfusion Fast-Step, Warner Instruments), using a gravity driven sewer-pipe perfusion system, followed by a 60 second rinse interval. All experiments were performed at room temperature and currents were recorded using a Dagan amplifier, acquired at 1 kHz and stored on computer for later analysis (pClamp5).

Peptide release experiments

Following loading of the terminals onto filters they were perfused with NL saline at an initial rate of 200 $\mu\text{L}/\text{min}$ which was then slowly increased to 750 $\mu\text{L}/\text{min}$. Following a stabilization period of 30 minutes samples were then collected every 24–30 seconds prior to, during and subsequent to ATP stimulation. The P2X receptor antagonist, Pyridoxal-phosphate-6-azophenyl-2, 4 -disulfonic acid (PPADS; 3 minutes) or Brilliant Blue-G (BBG; 30 minutes) were perfused prior to the challenge with ATP and continued throughout the duration of the ATP stimulation. In the experiments using 0 mM NaCl, the solutions were prepared by replacing NaCl with an equimolar concentration of N-methyl-D-glucamine chloride (NMDG-Cl) and were exchanged prior to the stabilization period. The AVP content of each perfusate sample was determined using a specific AVP Enzyme-linked Immunoassay Kit (Assay Designs, Ann Arbor, MI). Significance between treatments was determined using a t-test. $p < .05$ was considered statistically significant. Results are expressed as the mean $\pm$ SE.

MATERIALS

ATP, PPADS and BBG were purchased from Sigma-Aldrich (Saint Louis, MO).

RESULTS

ATP-dependent currents

Exposure of isolated NHTs to 50 μM ATP induces fast activating, slowly inactivating inward currents (Fig. 1). A large current component (91%) is Na^+ -dependent while Ca^{2+} is the main permeating cation in the remaining current (Fig. 1A). The amount of extracellular calcium has a significant impact on the current magnitude. Increments from normal (2.2 mM) extracellular $[\text{Ca}^{2+}]_o$ decrease total current by 64%, while lowering $[\text{Ca}^{2+}]_o$ enhances total current by 31% (Fig. 1B). Calcium directly affects Na^+ conduction through open P2X channels as demonstrated by the decrease in the fast activating, slowly inactivating portion of the current (Fig. 1B). Current-voltage plots, obtained at various membrane potentials, present a clear rectification pattern in the ATP-dependent current (Fig. 1C). Comparison (11) of the normalized inward currents indicates that rectification is dependent on both Na^+ and Ca^{2+} (Fig. 1D) and also reveals differences in reversal potentials (see discussion).

ATP-dependent neuropeptide release

To determine the effect of extracellular calcium and/or sodium on ATP-stimulated AVP release, rat NHT were perfused with normal Locke's buffer, sodium-free Locke's buffer, or calcium-free Locke's buffer. In NL buffer, a 2 minute stimulation with ATP (200 μM) caused an initial transient increase in AVP ($80.09 \pm 5.28\%$) followed by a sustained release of AVP that lasted throughout the duration of the ATP stimulation (Fig. 2A and D). In sodium-free Locke's buffer a 2-minute stimulation with ATP (200 μM) also caused an initial transient increase in AVP ($212.95 \pm 9.63\%$) but abolished the sustained AVP release induced by ATP in NL buffer (Fig. 2B and D). Furthermore, removal of only extracellular calcium resulted in no ATP-induced transient increase in AVP release, but had no effect on the delayed, sustained increase in AVP release induced by ATP (Fig. 2C and D).

ATP-receptor types

To determine which receptor subtypes might be responsible for the different release components, the effect of selective P2X receptor antagonists on the calcium and sodium dependent ATP evoked AVP release were tested. NHTs were pretreated with PPADS or BBG prior to ATP stimulation. The ATP-induced calcium-dependent transient increase in AVP release was significantly ($p < 0.01$; Fig. 3A and B) reduced, by 93%, with 10 μM of the P2X purinergic receptor antagonist PPADS compared to untreated control (4.73 ± 7.34 versus $75.30 \pm 13.80\%$ increase, respectively). This is a dose that can block P2X2 and P2X3 receptors but not P2X4 or P2X7 receptors, all of which are present on rat NHT (11). Importantly, the ATP-induced calcium-independent, sodium-dependent sustained increase in AVP release was not affected ($p > 0.5$; Fig. 3A and B) by pretreatment with PPADS.

Pretreatment of rat NHTs with the selective P2X7 receptor antagonist BBG (100 nM) had no effect ($p > 0.5$) on the ATP-induced calcium-dependent transient increase in AVP release

compared to untreated controls (165.01 ± 61.16 vs. $147.03 \pm 27.00\%$ increase, respectively; Fig. 4A and C). However, the ATP-induced sodium-dependent sustained increase in AVP release was significantly ($P < 0.01$) reduced, by 55%, by pretreatment with BBG (100 nM) compared to untreated controls (61.56 ± 9.02 versus $135.88 \pm 14.37\%$ increase, respectively; Fig. 4B and C).

To confirm that the sustained sodium-dependent AVP release component is mediated by P2X7 receptors, we exposed terminals to both a dose (10 μ M) of ATP that can activate P2X2 and P2X3 but not P2X7 receptors, and a dose (100 μ M) effective for all P2X receptors (Fig. 5). In the absence of extracellular calcium, 100 μ M ATP resulted in a sustained increase of AVP release (see Fig. 2C), whereas 10 μ M ATP had no effect on AVP release. No ATP-induced calcium-dependent transient increase in AVP was observed, as expected, with either dose (Fig. 5).

DISCUSSION

ATP-receptor subtypes P2X2, P2X3, P2X4 and P2X7 are found in AVP-containing NHT (12). However, only P2X7 receptors are found in OT-containing NHT (3). All of these purinergic receptor types are known to be cation selective (22). The permeant ions for P2X receptors are ordered in selectivity according to Eisenman's sequence IV ($K^+ > Rb^+ > Cs^+ > Na^+ > Li^+$), and the channels are essentially impermeant to NMDG, Tris, and tetraethylammonium (1, 22).

Here we have shown that both Na^+ and Ca^{2+} are permeable through these NHT receptors (see Fig. 1), but to varying degrees. That is, the ATP-induced current in NHT has both Na^+ and Ca^{2+} components, with Na^+ being by far the largest component (Fig. 1A). Furthermore, Ca^{2+} inhibits the ATP-induced Na^+ current (Fig. 1B). This agrees well with previous findings (22) that extracellular divalent cations block the open channel; with the EC_{50} for calcium ~ 5 mM. Divalents also reduce the probability of a channel being open; with the EC_{50} for calcium ~ 1.3 mM. At the whole cell level, the currents induced by ATP also show strong inward rectification (Fig. 1C). Ca^{2+} and Na^+ (Fig. 1D) but not Mg^{2+} (not shown) are responsible for the rectification of the ATP-induced current. Its persistence in divalent-free solutions indicates that this rectification does not simply result from block of the permeation pathway by divalent cations.

In accordance with the above permeabilities, we determined whether Na^+ and/or Ca^{2+} might contribute to the ATP-stimulated release of AVP from NHT (Fig. 2). High concentrations of ATP in NL buffer resulted in an initial transient release of AVP followed by a sustained release of AVP (Fig. 2A). Replacing NaCl with NMDG-Cl had no effect on the ATP-induced transient increase in AVP (Fig. 2B) release, but diminished the sustained release of AVP (Fig. 2B). Removal of extracellular calcium had no effect on the ATP-induced sustained release of AVP (Fig. 2C) but completely abolished the ATP-induced transient increase in AVP (Fig. 2C). It also appears as if the removal of sodium enhances the transient response and the removal of calcium seems to enhance the sustained response (Fig. 2D). Thus, the ATP-induced initial increase in AVP release was calcium-dependent and the late increase in AVP release was calcium-independent but sodium-dependent.

Given that the known NHT purinergic receptors have different Ca^{2+} vs Na^{+} permeabilities and calcium fractional percentages (19, 22), we tried to determine which subtype(s) might be responsible for the observed currents and neuropeptide release components (Fig. 3). The ATP-induced inactivating current in NHT has been shown to be sensitive to the P2X2 and P2X3 receptor antagonist PPADS, whereas, the ATP-induced sustained current was insensitive to PPADS (12). The calcium-dependent ATP-induced release was sensitive to PPADS (Fig. 3A and 3B), whereas, the sodium-dependent ATP-induced release was insensitive to PPADS (Fig. 3A and 3B). Thus, this initial component of AVP release appears to be due to P2X2 and P2X3 receptors (Fig. 3B). The role of P2X2/3 receptors has also been demonstrated by specific knock-outs on ATP-stimulated release (6).

Since BBG, at 10 nM, has been shown to block only non-inactivating ATP currents (3), we used this P2X7 receptor antagonist to determine if the late component of release was mediated by this receptor type (Fig. 4). BBG did not inhibit the Ca^{2+} -dependent transient release (Fig. 4A) but did inhibit the late sustained Na^{+} -mediated response (Fig. 4B). Furthermore, the sodium-dependent ATP-induced release of AVP was dependent on the dose of ATP (Fig. 5). The currents through P2X7 receptors, in contrast to those observed at other P2X receptors, require concentrations of ATP greater than 100 μM to be activated (22). Thus, this late component of AVP release appears to be due to the P2X7 receptor.

P2X2 receptors are permeable to calcium with their $P_{\text{Ca}}/P_{\text{Na}}$ approximately equal to 2.5 (8). This is less than P2X4 receptors but more than P2X3 receptors (19) as shown in other systems. P2X4 receptors are channels with a calcium permeability that is relatively high (~4.2). However, the calcium contribution by this receptors is ~8% of the total inward current under normal conditions. In contrast, the Ca^{2+} vs. Na^{+} permeabilities and fractional percent of current for P2X7 receptors are 0.7 and 2.8%, respectively (22). Thus, the fractional current and permeability of Ca^{2+} vs. Na^{+} is highest for the P2X2 and P2X4 receptors and lowest for the P2X7 receptor channel.

Our I-V results (Fig. 1D) demonstrate that a change in reversal potential (V_{rev}) equal to +12 mV is obtained when calcium is removed from NL. Using a previously published (8) relative permeability equation for the total ATP-elicited current, then

$$P_{\text{Ca}}/P_{\text{Na}} = \exp(\Delta V_{\text{rev}} * F/RT) = 2.01$$

This estimation provides a $P_{\text{Ca}}/P_{\text{Na}}$ lower than expected for P2X2, P2X3 or P2X4 but higher than for P2X7 alone. Thus, the latter purinergic receptor, even in AVP-terminals, appears to be contributing in some measure to Ca^{2+} permeability. Regrettably, it is not straightforward to make an accurate measurement of the Ca^{2+} permeability of the P2X receptors, because of the current block that Ca^{2+} causes (see Fig. 1B). In any case, the differential effects of ATP on neuropeptide release cannot be explained solely by these properties, however, but by, perhaps, receptor localization and utilization of different intra-terminal release mechanisms (17). It is also important to emphasize again that Na^{+} can cause release independently of Ca^{2+} (25).

Therefore, these results show that rat isolated NHT exhibit a biphasic response to exogenous ATP that is differentially dependent on extracellular calcium vs. sodium influx. The initial transient release appears to be through P2X2 and/or P2X3 receptors and is dependent on extracellular calcium, while the sustained release seems to be mediated by P2X7 receptors and is dependent on extracellular Na⁺. Thus, these receptors might be localized not only to different terminal types but also to different areas of the same terminals. P2X2 and/or P2X3 receptors could be co-localized with release sites and thus Ca²⁺ entry through them causes release. In contrast, P2X7 receptors could be localized away from release sites, so that only Na⁺ entry through them causes release via a different mechanism (25). The role of P2X4 receptors is questionable since none of the blockers used in this or other (12) studies affect its function.

In conclusion, P2X2 and/or P2X3 receptors potentiate AVP release through the influx of calcium through their channels, while P2X7 receptors can only affect AVP release at higher concentrations of ATP and via influx of sodium through its channels (17). Importantly, extracellular Ca²⁺ is depleted during bursting stimulation of neuropeptide release from these NHT (20), so these differences in dose, kinetics, and intracellular mechanisms could play an important role during such physiological activity (18). Since bursting also stimulates the release of many other transmitters, these differences might be important for synaptic function at other CNS terminals.

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Figure 1. Effects of extracellular Na^+ and Ca^{2+} on ATP-induced currents

Representative inward currents produced by 2–3 sec stimulations (see bars) with 50 μM ATP in control NL conditions. **A)** Replacing NaCl (0 Na^+) with NMDG-Cl in the presence of normal $[\text{Ca}^{2+}]_o$ reduces the inward current by 91%. An additional reduction (to 3 μM) of $[\text{Ca}^{2+}]_o$ almost completely abolishes the current. **B)** A reduction of the $[\text{Ca}^{2+}]_o$ from 2.2 mM to 3 μM in the presence of normal $[\text{Na}^+]_o$, increases the ATP-current by 31%. On the other hand, increasing the $[\text{Ca}^{2+}]_o$ to 10 mM reduces the ATP current by 64%. **C)** Depolarizing the membrane potential by 20 mV incremental steps in normal Na^+ plus 3 μM Ca^{2+} Locke's results in the gradual elimination of the ATP-induced current, indicative of an inward rectifying current. **D)** Normalized comparisons of the inward rectifying current indicates that rectification is dependent on both Na^+ and Ca^{2+} . Hollow squares – Normal Locke's (NL) with 140 mM Na^+ and 2.2 mM Ca^{2+} , filled squares – NL with 140 mM Na^+ and 3 μM Ca^{2+} , filled circles – NL 0 mM Na^+ and 2.2 mM Ca^{2+} , and hollow circles – NL with 0 mM Na^+ and 3 μM Ca^{2+} . NL with 140 mM Na^+ and 2.2 mM Ca^{2+} with 0 Mg^{2+} produced the same results as NL with 140 mM Na^+ and 2.2 mM Ca^{2+} with 1 mM Mg^{2+} (4). $n=3-4$ and $\text{HP}=-80$ mV for all experiments.

Figure 2. ATP-induced AVP release from rat isolated NHT is differentially affected by extracellular calcium and sodium

In rat isolated NHT, ATP (200 μM) perfusion in NL buffer (145 mM NaCl; 2.2 mM CaCl₂) resulted in an initial transient release followed by a sustained release of AVP (graph A,D). Replacing NaCl with NMDG-Cl (145 mM NMDG-Cl; 2.2 mM CaCl₂) had no effect on the ATP-induced transient increase in AVP (graph B,D) release, but diminished the sustained release of AVP (graph B,D). Removal of extracellular calcium (145 mM NaCl; 0 mM CaCl₂) had no effect on the ATP-induced sustained release of AVP but completely abolished the ATP-induced transient increase in AVP release (graph C,D). Figure D represents an overlay of the ATP-stimulated AVP release in NLs (solid circles), zero sodium (open circles) and zero calcium (plus sign). AVP content is expressed as a percentage of the basal release prior to exposure to ATP. Data are the mean ± SE for AVP (percent of basal) at each time point. Number of filters are 3–5 for each figure. Basal release of AVP for Normal Locke's, zero calcium and zero sodium Locke's buffer groups was 62.35 ± 8.15, 44.45 ± 4.26 and 20.43 ± 1.25 pg/ml, respectively.

Figure 3. Effect of PPADS on ATP-induced AVP release from isolated NHT

Rat NHT were perfused with NL saline (solid circles; $n=3$) or pretreated with 10 μM PPADS in NL buffer (open circles; $n=3$) prior to and during a 5 minute stimulation with ATP (200 μM). PPADS (10 μM) had no effect on basal release of AVP (graph A). AVP content is expressed as a percentage of the basal release prior to exposure to ATP. Data are the mean $\pm$ SE for AVP at each time point. B) Bar graph summarizing the effects of PPADS on ATP-induced AVP release. In the presence of PPADS, the ATP-induced calcium dependent transient peak of AVP release was significantly ($p<0.01$,) reduced by 93% compared to untreated controls. However, the ATP-induced sodium dependent sustained release of AVP was not affected ($p>0.5$) by pretreatment with PPADS. Data is given as the mean area under the curve for the 3 highest points during the ATP stimulation with and without PPADS. Significance between treatments was determined using a t-test. Basal release of AVP for control and PPADS treatment groups was 39.15 ± 0.17 and 42.28 ± 6.80 pg/ml, respectively.

Figure 4. Effect of BBG on ATP-induced AVP release from isolated NHTs

A) Rat isolated NHT were perfused with only NL buffer (solid circles; $n=3$) or pretreated for 30 minutes with 100 nM BBG, a P2X7 receptor antagonist in NL (open circles; $n=3$) prior to and during an 8 minute stimulation with ATP (200 μ M). BBG (100 nM) had no effect on basal release of AVP. **B)** Rat NHT were perfused with zero calcium Locke's buffer (solid circles; $n=3$) or pretreated with 100 nM BBG in zero calcium Locke's buffer (open circles; $n=3$) prior to and during an 8 minute stimulation with 200 μ M ATP. AVP content is expressed as a percentage of the basal release prior to exposure to ATP. Data are the mean $\pm$ SE for AVP at each time point. **C)** Bar graph summarizing the effects of BBG on ATP-induced AVP release. In the presence of BBG, the ATP-induced sodium dependent sustained release of AVP release was significantly ($p<0.01$) reduced by 55% compared to untreated controls. However, the ATP-induced calcium dependent transient release of AVP was not significantly ($p>0.5$) affected by pretreatment with BBG. Significance between treatments was determined using a t-test. Basal release of AVP in NL's buffer for control and BBG treatment groups was 8.67 ± 0.96 and 7.19 ± 1.00 pg/ml, respectively. Basal release of AVP in zero calcium Locke's buffer for control and BBG treatment groups was 4.16 ± 0.46 and 7.90 ± 0.53 pg/ml, respectively.

Figure 5. Effect of low vs. high concentrations of ATP on the sustained release component of AVP
 Rat isolated NHT were perfused with zero calcium Locke's buffer and stimulated for two-minutes with 10 μ M ATP, then, following a 5-minute rest period, were stimulated for two-minutes with 100 μ M ATP. AVP (solid circles; $n=3$) concentrations were determined in perfusate samples collected every 24 seconds prior to, during, and after stimulation with 10 and 100 μ M ATP. In the absence of extracellular calcium 10 μ M ATP had no effect on AVP release, whereas, 100 μ M ATP resulted in a sustained increase of AVP. No ATP-induced transient increase in AVP was observed at either dose. AVP content is expressed as a percentage of the basal release prior to exposure to ATP. Data are the mean $\pm$ SE for AVP at each time point. Basal release of AVP prior to ATP treatment was 30.96 ± 1.25 pg/ml.