Simultaneous exposure to ATP and phenylephrine induces a sustained elevation in the intracellular calcium concentration in supraoptic neurons

Zhilin Song, Sukumar Vijayaraghavan, and Celia D. Sladek
Department of Physiology and Biophysics, University of Colorado at Denver and Health Sciences Center, Aurora, Colorado

Submitted 11 October 2005; accepted in final form 23 January 2006

pressin (VP) release from the hypothalmo-neurohypophyseal system (HNS) is stimulated by ATP activation of P2X purinergic receptors and by activation of α1-adrenergic receptors by phenylephrine (PE). These responses are potentiated by simultaneous exposure to ATP+PE. Potentiation was blocked by depleting intracellular calcium stores with thapsigargin. To test the hypothesis that the synergistic response to ATP+PE reflects alterations in the intracellular calcium concentration ([Ca2+]i), [Ca2+]i was monitored in supraoptic neurons in HNS explants loaded with fura 2-AM. Both ATP and PE induced rapid, but transient, elevations in [Ca2+]i. In 0.3 mM Ca2+, the peak response to ATP was greater than to PE but did not differ from the peak response to ATP+PE. A sustained elevation in [Ca2+]i, was induced by ATP+PE, that was greater than ATP or PE alone. In 2 mM Ca2+, the peak response to ATP+PE was significantly greater than to either ATP or PE alone, and the sustained response to ATP+PE was greater than to either agent alone. Responses were comparable in the presence of TTX. The sustained elevation in [Ca2+]i, was also observed when ATP+PE was removed after 1 min, but it was eliminated by either thapsigargin or removing external calcium, indicating that both calcium influx and calcium release from internal stores are required. Some cells were vasopressinergic based on a VP-induced increase in [Ca2+]i. These observations support the hypothesis that simultaneous exposure to ATP+PE induces a different pattern of [Ca2+]i than either agent alone that may initiate events leading to synergistic stimulation of VP release.

vasopressin; oxytocin; norepinephrine; posterior pituitary; fura-2AM

VASOPRESSIN (VP) RELEASE from the posterior pituitary is stimulated by a decrease in blood volume or pressure (25). Although information about moderate decreases in blood volume and/or pressure is transmitted to the neurons in the supraoptic nucleus (SON) and paraventricular nucleus (PVN) via the A1 noradrenergic pathway (4, 12, 30, 31), adrenoreceptor antagonists did not block A1 activation of VP cells, suggesting that these neurons use agents in addition to norepinephrine (NE) for neurotransmission (5). The finding that application of the P2 purinergic receptor antagonist, suramin (10 mM) in the SON reversibly blocked excitation of VP neurons by A1 stimulation without preventing the excitatory effect of locally applied NE (6, 7) suggested that in addition to NE, this pathway also uses ATP as a neurotransmitter. Local suramin injections in SON also reduced Fos expression induced by moderate hemorrhage, but not by osmotic stimulation in SON neurons (3). In studies designed to determine the importance of corelease of ATP and NE from this A1 pathway on VP release, it was found that simultaneous exposure of explants of the hypothalmo-neurohypophyseal system (HNS) to ATP and phenylephrine (PE), an α1-adrenergic receptor agonist, resulted in synergistic stimulation of VP release. The synergistic response was larger than that observed with ATP or PE independently, and in contrast to the transient responses observed in response to independent delivery of ATP or PE, was sustained for several hours (17). The sustained component was blocked by actinomycin, indicating that new gene transcription is required (17). Because additional experiments implicated the calcium permeable ionotropic P2X-purinergic receptors in the ATP response (17) and because activation of α1-adrenergic receptors activates inositol triphosphate and releases calcium from intracellular stores (9), the current experiments tested the hypothesis that the synergistic response to ATP+PE reflected convergence of these signaling cascades to increase intracellular calcium.

To test the hypothesis, the effect of depleting intracellular calcium stores with thapsigargin (TG) treatment on the synergistic action of ATP+PE on VP release from HNS explants was examined. However, it was not possible to use VP release to evaluate the effect of calcium influx, because exocytosis is dependent on calcium influx. Therefore, changes in intracellular calcium ([Ca2+]i) were measured using the calcium sensitive dye, fura-2AM.

Although the effect of ATP on [Ca2+]i in dissociated SON neurons has been reported previously (24), the current studies extend these observations to evaluate the interaction between ATP and PE and use an organotypic preparation, the HNS explant, rather than dissociated neurons for calcium imaging. The latter is important, because in the HNS explant, neuronal/glial relationships are preserved, as well as local neuronal circuitry. The explant is also advantageous, because it is the same preparation in which the synergistic effect of ATP+PE on VP and oxytocin (OT) release was observed. Therefore,
observed changes in $[\text{Ca}^{2+}]$, could induce calcium-dependent mechanisms that drive new gene transcription and protein synthesis that sustains the effect of ATP+PE on hormone release. The effect of ATP+PE on $[\text{Ca}^{2+}]$, was examined under four distinct conditions: extended (4 min) exposure to ATP+PE in low extracellular calcium ($[\text{Ca}^{2+}]_o$; 0.3 mM), normal $[\text{Ca}^{2+}]_o$ (2 mM), and in the presence of TTX, and after short (1 min) exposure to ATP+PE. Low $[\text{Ca}^{2+}]_o$, was used to replicate the conditions of the hormone release studies. However, because low $[\text{Ca}^{2+}]_o$ increases the excitability of SON neurons by increasing the amplitude of the persistent Na current ($I_{\text{NaP}}$) (19), it was important to determine whether the effects of ATP+PE were dependent on $[\text{Ca}^{2+}]_o$, TTX was used to determine whether the effects of ATP+PE were dependent on $I_{\text{NaP}}$ or on transient Na current (e.g., action potential generation). The latter could reflect activation of local neuronal circuitry by ATP and/or PE.

**MATERIALS AND METHODS**

**Animals**

Male Sprague-Dawley rats (Crl:CD(SD)Br; Charles Rivers Laboratories, Wilmington, MA) were used in all experiments. 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 at Denver and Health Sciences Center.

**HNS Explant Perfusion Experiments**

Explants of the HNS were obtained from decapitated male Sprague-Dawley rats (125–150 g) as described previously (28). They were dissected from the base of the hypothalamus and included the VP neurons of the SON with their axonal projections extending through the median eminence and terminating in the neural lobe. They also included the organum vasculosum of the lamina terminalis, and the arcuate nucleus, but not the PVN. Explants were perfused individually in closed culture chambers (0.5 ml) at 37°C with oxygenated culture medium at a rate of 2.0 ml/h. The medium consisted of F12 nutrient mixture (Sigma, St. Louis, MO) fortified with 20% fetal bovine serum, 1 mg/ml glucose, 50 mM penicillin, 50 mg/ml streptomycin. Bacitracin (0.28 mM) was added to the medium to prevent degradation of VP (26). The explants were equilibrated for 4 h and then maintained under different treatment conditions for an additional 4 to 5 h. Outflow was collected individually at 20-min intervals using a refrigerated fraction collector maintained at 4°C.

**Experimental Design**

**Experiment 1.** To evaluate the effect of TG alone on basal VP release, explants were treated with TG (200 nM) or vehicle (DMSO) for 0.5 h after 4-h equilibration. TG was dissolved in 50 μl DMSO, and this was diluted to 50 ml.

**Experiment 2.** To evaluate the effect of depleting the intracellular $\text{Ca}^{2+}$ stores on the response to ATP+PE, explants were treated with TG (200 nM) or vehicle (DMSO) for 0.5 h after 4-h equilibration and after 0.5-h wash, both groups were exposed to ATP/PE (100 μM each) for another 4 h. The medium for both groups contained 0.03% ascorbic acid to prevent PE oxidation.

**Radioimmunoassay.** VP concentration in the perfusate was determined by RIA, as described previously (29), using antisera against VP (Arnel Products, Brooklyn, NY) at a final dilution of 1:100,000. The buffer used was 0.1 M PBS, pH 7.6, with 1 mg/ml BSA and 1 mg/ml sodium azide. Both standards and samples were incubated for 72 h at 4°C in the presence of 5,000 cpm of $^{125}$I-labeled arginine VP (NEN, DuPont). Antibody-bound VP was separated from free peptide with dextran-coated charcoal, and the amount of $^{125}$I-labeled VP in the pellet was determined with a γ-counter. The assay has a minimum sensitivity of 1.0 pg/tube and a useful range up to 100 pg/tube.

**Data analysis.** Basal hormone release was determined for each explant as the hormone release at the end of the 4-h equilibration period. Subsequent hormone release is expressed as a percentage of this basal value. In each experiment, two-way ANOVA with repeated measures and post hoc test (Student-Newman-Keuls) was used to determine the specific group differences at individual time points. The level of significance was set to $P < 0.05$. Results are expressed as the means ± SE.

**Calcium Imaging**

**Dye loading.** The same HNS explant preparation, prepared from 125–150 g male Sprague-Dawley rats was used for live cell $\text{Ca}^{2+}$ imaging. The acetoxyethyl (AM) ester form of fura-2 (Molecular Probes, Carlsbad, CA; 50 μg) was dissolved in 50 μl of anhydrous DMSO to make a 1 mM stock solution. Explants were incubated in 100 μM fura-2-AM for 10 min followed by a 50 min incubation in 20 μM fura-2-AM in medium containing 0.02% pluronic acid F-127 (Molecular Probes). Dye loading and imaging were performed in F12 nutrient medium (Sigma) fortified with 1 mg/ml glucose, and (1.7 mM CaCl$_2$ in some experiments), and gassed with 95%O$_2$-5%CO$_2$.

**Perfusion and drug delivery.** Explants were placed in a chamber fitted onto the stage of an upright fluorescent microscope. The chamber was formed by an adaptor (Bioscience Tools, San Diego, CA) placed in a 35 mm Corning petri dish whose base was coated with Sylgard (1 mm). The explant was stabilized with a weighted, ring-shaped stainless steel net, which was pinned onto the Sylgard base. Gassed medium was delivered by a gravity-driven 8-valve system (Warner Instruments, Hamden, CT) to the perfusion port at a rate of ~3 ml/min. and was removed by a vacuum port at the opposite side.

**Fluorescence ratio imaging.** The explant was positioned in the recording chamber with its ventral side up, stabilized with a weighted nylon mesh, and allowed to equilibrate for 1 h. Thus the optic chiasm is easily visualized, and the SON can be located anatomically in the tissue immediately rostral and parallel to optic chiasm. Fura-2 loaded magnocellular neurons were alternatively excited with 340 nm and 380 nm UV light from a Xenon source (Sutter Instruments, Novato, CA). Exposure time for 380-nm wavelength was between 200 and 500 ms and was tripled for 340 nm wavelength accordingly. 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 charged-couple device camera (Hamamatsu, Japan). Paired 340/380 excitation images were acquired every 3 s using SlideBook software (Intelligent Imaging, Denver, CO) for a period of 100 frames. Explants treated with 10 μM ionomycin to allow $[\text{Ca}^{2+}]$, to equilibrate with extracellular $\text{Ca}^{2+}$ gave an apparent $R_{\text{max}}$ of 1.0684 ± 0.001 ($n = 15$) in 0.3 mM $\text{Ca}^{2+}$ and 2.0643 ± 0.0028 ($n = 16$) in 2 mM $\text{Ca}^{2+}$. As both values far exceed the highest ratio achieved by all agents studied in all experimental preparations, the peak responses reported were not limited by the maximum detectable change in the 340/380 ratio. The ratio data are presented as a percentage of the basal 340/380 ratio for each cell (see below).

**Data analysis.** Magnocellular neurons were identified by the size of the cell body (>25 μm in diameter), their location, and for some, their response to VP. Data from the identified neurons were collected as the average ratio of fluorescence from 340 nm excitation over fluorescence from 380 nm excitation from a region of interest that included the complete area of the cell body, including the nucleus if it were (rarely) evident at the focal plane of the image. This ratio is a direct, linear measurement of $[\text{Ca}^{2+}]$, within physiological ranges (14). A baseline ratio value for each neuron was determined by averaging ratios of the 10 frames preceding drug application. Responses were then expressed as percentage changes in 340/380 ratio relative to basal. The end ratio was calculated by averaging the percentage ratios
for frames 90–100 for each neuron. The mean ± SE of the percentage values from individual neurons were calculated and plotted. Parametric one-way ANOVA (F ratio) or Kruskal-Wallis one-way ANOVA on ranks (H value) were used to determine whether there were significant group differences in the peak response and the end ratio for ATP alone, PE alone, or ATP+PE. Paired Student’s t-test analysis was used to determine whether the end-ratio was different from the initial basal ratio for any treatment group.

RESULTS

Perfusion Experiments: Effect of TG.

Effect of TG treatment on basal VP release. VP release from HNS explants is stimulated by increases in osmolality (27, 35). Therefore, the increase in osmolality induced by the use of DMSO as the vehicle for TG, caused a transient increase in VP release that was observed in both the control and TG groups (Fig. 1A). TG treatment did not consistently alter the DMSO-induced VP release (Fig. 1A and B). Depletion of the intracellular Ca^{2+} store by TG is irreversible. Therefore, it and the vehicle DMSO were washed out after a 30-min exposure. With the return of osmolality to the basal level, subsequent VP release was not significantly different between the control and TG groups (Fig. 1A).

Effect of TG treatment on VP response to ATP+PE. In the absence of TG treatment, ATP+PE induced a sustained increase in VP release that characterizes the synergistic response to ATP+PE ($F_{\text{time}} = 3.277, P < 0.001$). Depletion of intracellular Ca^{2+} stores by TG treatment completely prevented this sustained stimulation of VP release by ATP+PE ($F=25.268$, $P < 0.001$) (Fig. 1B). This result supports the hypothesis that mobilization of intracellular Ca^{2+} is required for synergistic stimulation of VP release by ATP+PE.

Calcium Imaging

Identification of SON neurons for calcium imaging. Two approaches were useful in identifying fura-2 loaded cells as SON neurons.

LOCATION IN EXPLANT. Imaging was performed from the ventral surface of the HNS explant. SON is located immediately rostral and parallel to the optic chiasm (see Fig. 2A) which is easily visualized as a dark shadow when the explant is viewed under either bright light or UV illumination and serves as an excellent landmark for localizing SON. Fig. 2B shows the position of one imaged area and its relation to the optic chiasm. Neuronal size (>25 μm in diameter) and shape were also used to confirm that the fura-2 loaded cells were the magnocellular SON neurons. At high power (Fig. 2C), the characteristic bipolar/tripolar shapes of the large SON neurons are evident and neurites are seen to emanate from the cell body.

RESPONSE TO VP. Vasopressinergic neurons express V1a VP receptors and respond to VP with an increase in [Ca^{2+}], whereas oxytocinergic neurons express oxytocin receptors and respond to oxytocin with an increase in [Ca^{2+}]. These responses have been used to identify VP and OT neurons in dissociated cell preparations (8, 16). As shown in Fig. 3, in a single imaged field in one HNS explant, five neurons showed an increase in [Ca^{2+}] when exposed to VP, identifying these neurons as vasopressinergic and confirming that the localization procedure used above identifies SON neurons. Because of the expense of using VP or OT to identify SON neurons, this approach was not routinely used in all explants.

Calcium Responses to ATP, PE, or ATP+PE. The 340/380 ratio was used as an index of [Ca^{2+}]. Under basal conditions, the 340/380 ratio for SON neurons ranged from 0.45 to 0.77. To analyze changes in [Ca^{2+}], the basal ratio was determined for each cell by averaging the 10 ratio measurements (frames) immediately preceding addition of the test agent, and responses to the agent were expressed as a percentage of this average basal ratio (% of basal).

As shown in Figs. 3 and 4, exposure of explants to ATP (200 μM) resulted in a rapid increase in [Ca^{2+}], that reached a peak within 25–60 s and then quickly declined to a plateau level that was sustained throughout the period of exposure to ATP (3–4 min). Fig. 4 shows the response to ATP in one preparation. Pseudocolor images depicting the 340/380 ratio indicative of the basal [Ca^{2+}], and the peak change in [Ca^{2+}], are shown in Fig. 4A and B, respectively. Fig. 4C shows the time course of the change in the 340/380 ratio for individual cells imaged in a preparation treated with ATP, and Fig. 4D shows the 340 and 380 fluorescence records for three representative cells. It is
evident that individual cells show slight variation in the time to reach the peak. This may reflect differences in the diffusion distance to cells that are deeper in the preparation. Variability in the peak response is also evident, and spontaneous elevations in \([\text{Ca}^{2+}]_i\) are present both before and after ATP exposure in some cells. During the period preceding drug application, spontaneous peaks in \([\text{Ca}^{2+}]_i\) were observed in 19% of 114 neurons imaged in six different explants. Treatment of the explants with TTX before imaging reduced the occurrence of spontaneous spikes during the basal period to 8.6% (10 out of 116 cells in six different explants.)

Figure 5 shows the time course of responses to ATP, PE, and ATP/PE (200 \(\mu M\) each) in 0.3 mM \([\text{Ca}^{2+}]_i\). The responses of multiple neurons in a single visual field were analyzed in 3 or 4 separate explants per drug treatment group. Because subsequent drug exposures generally yielded smaller responses, the responses shown in Fig. 5 and analyzed in Fig. 6A represent the first drug exposure for each explant. As seen in Fig. 5, in 0.3 mM \([\text{Ca}^{2+}]_i\), the peak response to ATP and ATP/PE was similar in magnitude, but the peak response to PE was smaller (Fig. 6A; \(F = 7.483, P < 0.001\)). The plateau response to ATP and PE was similar and remained higher than basal \((P < 0.001)\), but combined exposure to ATP/PE resulted in a greater sustained elevation in \([\text{Ca}^{2+}]_i\). This sustained elevation in \([\text{Ca}^{2+}]_i\) was not due to loss of \([\text{Ca}^{2+}]_i\) homeostasis and cell death, because as seen in Fig. 3, cells were able to mount subsequent \([\text{Ca}^{2+}]_i\) responses. To analyze the sustained response, the “end ratio” was determined as the average of the last 10 ratios collected from each cell. The end ratio in response to ATP/PE was significantly larger than that to either ATP or PE alone (Fig. 6A; \(H = 35.264, P < 0.001\)). The larger sustained elevation in \([\text{Ca}^{2+}]_i\) may drive mechanisms responsible for the synergistic stimulation of hormone release by ATP/PE.

The data presented in Figs. 5 and 6A were obtained from experiments in which F-12 nutrient mixture was used as the perfusion medium to duplicate the conditions used in earlier hormone release experiments (17). However, the \([\text{Ca}^{2+}]_i\) concent-
tration in this medium was only 0.3 mM Ca\(^{2+}\). Therefore, to
determine whether the responses to ATP, PE, and ATP+PE
would be similar in 2 mM Ca\(^{2+}\) (the physiological extracellular
Ca\(^{2+}\)), the experiments were repeated in Fl2 nutrient mixture
supplemented with CaCl2. The peak ratios and end ratios for
experiments performed in 2 mM Ca\(^{2+}\) are shown in Fig. 6B. In
2 mM Ca\(^{2+}\), all elevations in [Ca\(^{2+}\)]\(_i\) were larger than those in
0.3 mM Ca\(^{2+}\) medium (peak ratios: \(P < 0.001\) for ATP, PE,
and ATP+PE; end ratios: \(P < 0.001\) for ATP, PE, and
ATP+PE). The peak ratio in response to ATP+PE was larger
than that to PE alone \((P < 0.001)\), as observed in low Ca\(^{2+}\).
The peak response to ATP+PE was also larger than the peak
response to ATP, but this was not observed in subsequent
experiments (see below and Figs. 6C and D). In 2 mM Ca\(^{2+}\),
extended exposure to ATP or PE alone resulted in sustained
elevations in [Ca\(^{2+}\)]\(_i\) compared with basal \((P < 0.001)\), but the
sustained elevation in [Ca\(^{2+}\)]\(_i\) was still larger in response to
ATP+PE compared with ATP or PE alone. The end ratios
followed this order: ATP+PE>PE>ATP \((H = 48.108, P < 0.001)\). Thus in both 0.3 mM and 2 mM external Ca\(^{2+}\),
the larger sustained elevation in [Ca\(^{2+}\)]\(_i\) in response to ATP+PE
could initiate events leading to synergistic stimulation of hor-
mone release by ATP+PE.

**Effect of TTX on responses to ATP, PE, and ATP+PE.** To
evaluate the role of efferent activity endogenous to the HNS
explant in the responses to ATP and PE, the above experiments
were repeated in the presence of TTX. Explants were pre-
treated with TTX (3 \(\mu M\)) for 5 min, and TTX was maintained in
the perifusate throughout exposure to ATP, PE, or
ATP+PE. Modified F-12 nutrient mixture with 2 mM Ca\(^{2+}\)
was used as the perifusate. The results are shown in Fig. 6C.
Neither the peak nor end responses to ATP and PE were
significantly different than those observed in 2 mM Ca\(^{2+}\)
without TTX (Fig. 6B). Both the peak and end responses to
ATP+PE were smaller in the TTX treated preparations
\((F=9.34, P = 0.003 \text{ and } F=11.83, P < 0.001, \text{ respectively})\),
but this may reflect unusually large responses to ATP+PE in
the explants analyzed in Fig. 6B, because when the peak
response data in Fig. 6B and D were combined, the difference
did not reach significance \((F= 3.012, P = 0.086)\). In the
TTX-treated explants, the peak response to ATP+PE was
greater than that to PE, but not significantly different from
that induced by ATP \((H = 18.249, P < 0.001)\). The end
ratio followed the same order as in the prior experiments in
2 mM Ca\(^{2+}\) without TTX treatment: ATP+PE>PE>ATP
\((H=28.984, P < 0.001)\). Thus TTX treatment did not change

Fig. 4. A and B: pseudocolor 340 nm/380 nm radiometric images (340 nm
assigned red; 380 nm assigned green) showing a representative example of
dye-loaded magnocellular neurons under basal conditions (A) and at their peak
response to ATP (B). C: time course of the change in 340 nm/380 nm ratio
for the response of neurons imaged in a preparation treated with ATP. Note the
spontaneous Ca\(^{2+}\) oscillations in cells 2, 3, 5, and 9 evident under basal
conditions in C. This experiment was performed in 2 mM Ca\(^{2+}\) without TTX.
ATP was added at the time indicated by the arrow. Variation in the responses
of individual cells is evident. Note that cell 6 shows a large peak response to
ATP followed by a sustained elevation in [Ca\(^{2+}\)]\(_i\) and a spontaneous Ca\(^{2+}\) peak
at the end of the recording. D: representative 340 and 380 traces from cells 1,
3, and 9 from C. Note the simultaneous increase in 340 and decrease in 380
fluorescence associated with application of ATP and during the spontaneous
Ca\(^{2+}\) oscillations.

Fig. 5. The time course of changes in [Ca\(^{2+}\)]\(_i\), induced by ATP, PE, and
ATP+PE in 0.3 mM Ca\(^{2+}\). Measurements of changes in the 340/380 ratio of
individual neurons within a single visual field were pooled from four explants
per group for ATP and PE and three explants for the ATP+PE group. For each
explant, the data are from the first drug exposure for that explant. ATP, PE,
or ATP+PE was added at the time indicated by the arrow. \(n\) (total number of
neurons) = 37 for ATP; 52 for PE; and 23 for the ATP+PE group.
the nature of the sustained responses to ATP, PE and ATP+PE.

Calcium responses to short application of ATP, PE, and ATP+PE. To determine whether the sustained elevation in the end ratio induced by ATP+PE required continued exposure to ATP+PE, the response to a short exposure to ATP, PE, or ATP+PE was evaluated. In this set of experiments, washout (3 min) of drugs was initiated 1 min after the peak of the response. With a perfusion chamber volume of 1 ml, the concentration of the drugs would be reduced 20-fold within 1 min and reduced to 1 nM by 4 min. As seen in Fig. 6D, the end ratios were significantly lower in all groups than observed when the drugs were present throughout the experiment (compared to Fig. 6B: ATP, $P < 0.001$; PE, $P < 0.001$; ATP+PE, $P = 0.004$), but they still followed the same order as observed with continued exposure: ATP+PE>PE>ATP ($H = 40.928$, $P < 0.001$).

Source of the $[Ca^{2+}]_i$, signal induced by ATP+PE. To determine the source of the peak and sustained elevation in $[Ca^{2+}]_i$ induced by ATP+PE, the responses to ATP+PE were examined after pretreatment of the fura-2-loaded preparation with TG (200 nM) for 30 min, in perfusion medium containing no CaCl₂ (plus 1 mM EGTA), or with both treatments (TG in zero CaCl₂; Fig. 7). Fig. 7A shows the time course of responses to ATP+PE of cells in a single preparation under these conditions. As shown in Fig. 7B, although removing external CaCl₂ only caused a slight, nonsignificant decrease in the initial peak, it prevented the extended elevation in $[Ca^{2+}]_i$; response. TG significantly reduced both the initial peak response and the sustained elevation in $[Ca^{2+}]_i$, (Fig. 7B). The delay to reach the peak response after TG treatment (Fig. 7A) may reflect more efficient CaCl₂ buffering as a result of TG treatment reducing basal $[Ca^{2+}]_i$. Finally, combined TG and zero CaCl₂, completely eliminated both the peak response and

Fig. 6. Comparisons of peak and end 340/380 ratios induced by ATP, PE, or ATP+PE in 0.3 mM CaCl₂ (A), 2 mM CaCl₂ (B), 2 mM CaCl₂ with TTX (C), and after a short drug exposure (D). A: in low CaCl₂ medium (0.3 mM, LCa), the ATP-induced peak ratio was greater than that induced by PE ($*P < 0.05$ ATP vs. PE) but not different from that induced by ATP+PE; the end ratio during continued exposure to ATP+PE was greater than that during exposure to either ATP or PE alone ($#P < 0.05$ ATP+PE vs. ATP or PE), although the end ratio remained significantly above basal for all three groups ($SP < 0.001$). B: in normal calcium (2 mM, NCa), the peak response to ATP+PE was greater than that to either ATP or PE alone ($*P < 0.05$ ATP+PE vs. ATP or PE); the end ratio during continued exposure to ATP+PE was greater than during exposure to either PE or ATP ($#P < 0.05$ ATP+PE vs. PE or ATP), although the end ratio remained significantly above basal for all three groups ($SP < 0.001$). Data were obtained from two explants per treatment. The total numbers of neurons analyzed were ATP, $n = 30$; PE, $n = 42$; ATP+PE, $n = 30$. C: in 2 mM CaCl₂ medium with TTX treatment (NCa,TTX), the peak responses to ATP and ATP+PE were greater than that to PE ($*P < 0.05$), but there was no difference between ATP and ATP+PE; the end ratio following ATP+PE was greater than PE or ATP; PE was also greater than ATP ($#P < 0.05$ vs. all other groups). Data were obtained from two explants per treatment. The total numbers of neurons analyzed were ATP, $n = 42$; PE, $n = 41$; ATP+PE, $n = 32$. D: drug washout was initiated 1 min after the peak response (NCa.S). The peak response to ATP+PE was greater than that of PE but not different from that of ATP ($*P < 0.05$, PE vs. ATP or ATP+PE). The end ratio followed the same order as in C: ATP+PE>PE>ATP ($#P < 0.05$ vs. all other groups). Data were obtained from two explants per treatment. The total numbers of neurons analyzed were ATP, $n = 36$; PE, $n = 35$; ATP+PE, $n = 35$. Therefore
the extended elevation in \([\text{Ca}^{2+}]\). Thus both \(\text{Ca}^{2+}\) influx and release of \(\text{Ca}^{2+}\) from internal stores contribute to the initial and the sustained \(\text{Ca}^{2+}\) responses to ATP+PE.

**DISCUSSION**

Although previous studies have used live cell \(\text{Ca}^{2+}\) imaging techniques to study the effect of glutamate, ATP, and neuropeptides on cytoplasmic \([\text{Ca}^{2+}]\) in SON neurons (8, 13, 15, 18, 22–24), to identify the types of \(\text{Ca}^{2+}\) channels used (22), and to study the role of \(\text{Ca}^{2+}\) in regulating the ionic currents in SON neurons (20, 21, 34), this is the first study to measure changes in \([\text{Ca}^{2+}]\), in response to ATP and an \(\alpha\)-adrenergic agonist in SON neurons maintained in an organotypic preparation. In this preparation, basal \([\text{Ca}^{2+}]\), was similar to that reported previously in isolated dispersed SON neurons (8) and in individual neurons in slice preparations containing SON (21, 34). The occurrence of spontaneous fluctuations in \([\text{Ca}^{2+}]\), was also similar to that observed in isolated SON neurons (8). These observations, in addition to the location, size, and responsiveness of a subpopulation of neurons to VP, supports the identification of the cells studied as SON neurons. In the preparations tested for responses to VP, 70% of the imaged neurons responded to VP. This probably reflects the selection of more ventrally oriented SON neurons for imaging, because VP neurons are preferentially located in the ventral portion of the nucleus. However, the 30% that were not VP responsive are probably oxytocinergic. Therefore, both OT and VP neurons are represented in the populations of neurons imaged. However, since the purpose of these studies was to examine the \([\text{Ca}^{2+}]\) response to combined exposure to ATP and PE, and this treatment induced similar synergistic stimulation of both VP and OT release from HNS explants (17), we did not feel it was necessary to identify the VP or OT phenotype of all neurons in this study. In addition, virtually 100% of the cells responded to ATP and/or ATP+PE without evidence of distinct subpopulations suggesting similar effects of ATP and ATP+PE on VP and OT neurons in SON.

The observation that combined exposure to ATP+PE resulted in an extended elevation in \([\text{Ca}^{2+}]\), that was significantly greater than that observed with either ATP or PE alone supports the hypothesis that the intracellular signaling cascades activated by ATP and PE converge to yield a greater sustained elevation in \([\text{Ca}^{2+}]\), than is achieved with either agent independently. This was observed under several different conditions, including low (0.3 mM) and normal (2 mM) extracellular \(\text{Ca}^{2+}\) both with and without TTX. Although smaller, it was even observed several minutes later when washout of ATP and PE was initiated one min after the peak response, and in at least one preparation (Fig. 3), it remained slightly elevated following a 30-min washout. The sustained increase in \([\text{Ca}^{2+}]\), induced by exposure to ATP+PE, although significantly greater than that observed with either agent alone, was not greater than the sum of the elevation observed to ATP or PE alone. Thus it probably reflects the additive effects of ATP+PE on \([\text{Ca}^{2+}]\). This and the observation that continued exposure to ATP+PE was not necessary to induce a sustained elevation in \([\text{Ca}^{2+}]\), indicate that an altered \([\text{Ca}^{2+}]\) signal persists that could initiate the events that lead to new gene transcription and synergistic stimulation of VP release.

Elevations in \([\text{Ca}^{2+}]\), alter a wide array of cellular functions that might initiate events leading to the extended elevation in VP release observed in response to exposure to ATP+PE. Specifically, in VP neurons, elevated \([\text{Ca}^{2+}]\), inactivates a \(\text{Ca}^{2+}\)-sensitive K⁺-current (\(I_{K,leak}\)) causing membrane depolarization and excitation (19). \([\text{Ca}^{2+}]\), also regulates the activity of \(\text{Ca}^{2+}\)-sensitive kinases such as PKC and \(\text{Ca}^{2+}\)-calmodulin kinase. This could lead to alterations in K⁺-channel activation (19), receptor trafficking, or gene expression by phosphorylation of transcription factors. The latter possibility is supported by the prior finding by this laboratory that actinomycin treatment blocked the extended elevation in VP release, which indicates that gene transcription is required for the response (17). Thus it is possible that the larger extended elevation in \([\text{Ca}^{2+}]\), although not in itself synergistic, initiates cellular processes, including new protein synthesis that convert a transient response into a response that, by virtue of its extended nature, is clearly synergistic (17). Future experiments in preparations that allow precise calibration of the fura signals to \([\text{Ca}^{2+}]\) (18, 21) will be useful for quantitatively assessing the potential for the observed changes in \(\text{Ca}^{2+}\) to alter channel or kinase activity.
A role for \([\text{Ca}^{2+}]\), in driving the synergistic response is
further supported by experiments that have eliminated several
other possible mechanisms. Alterations in ATP metabolism to
adenosine is not likely to participate, because inhibition of
ATP conversion to adenosine does not convert the transient
response to ATP to a sustained response (32). Modification of
stimulus secretory coupling at the nerve terminal was elimi-
nated in experiments using isolated neural lobes, and involve-
ment of glutamatergic afferents was eliminated in experiments
demonstrating that neither ionotropic nor metabotropic excit-
atory amino acid receptor antagonists block the synergistic
stimulation of VP release by ATP+PE (33).

The source of \([\text{Ca}^{2+}]\) for the extended elevation in \([\text{Ca}^{2+}]\), in
response to ATP+PE was evaluated by depleting internal \([\text{Ca}^{2+}]\)
stores with TG and by eliminating \([\text{Ca}^{2+}]\) from the perifusate.
Since P2X receptors are expressed in SON neurons (24), and
our prior studies with the P2X receptor antagonist, PPADS,
implicated P2X receptors in the synergistic response to
ATP+PE (17), influx of \([\text{Ca}^{2+}]\) through these nonselective
ation channels is expected to contribute to the response. The
requirement for an influx of external \([\text{Ca}^{2+}]\) was confirmed by
the absence of an extended elevation in \([\text{Ca}^{2+}]\), when
ATP+PE was applied in zero \([\text{Ca}^{2+}]\). Activation of \(\alpha\)-adrenergic
receptors induces production of inositol triphospho-
phate resulting in release of \([\text{Ca}^{2+}]\) from intracellular stores. The
sustained elevation in \([\text{Ca}^{2+}]\), was eliminated by depleting the
intracellular stores with TG, and the importance of intracellular
\([\text{Ca}^{2+}]\) stores to the synergistic response was demonstrated by
the ability of TG to block the extended increase in VP release
in response to ATP+PE. Thus the additive increase in \([\text{Ca}^{2+}]\),
after exposure to ATP+PE may reflect summation of these two
sources of intracellular \([\text{Ca}^{2+}]\). ATP and PE also depolarize
SON neurons initiating action potential firing (1, 16). This will
also initiate influx of \([\text{Ca}^{2+}]\) through the high voltage-activated
\([\text{Ca}^{2+}]\) channels (L-, N-, and P-type) that have been functionally
identified in SON neurons (10, 11) and have been shown to be
primarily responsible for the rapid increases in \([\text{Ca}^{2+}]\), in
response to action potentials (21). The sphere of influence of
these respective sources of \([\text{Ca}^{2+}]\) may determine their relative
contributions to induction of the extended increase in VP
release. In elegant modeling studies, Roper et al. (21) have
provided evidence supporting roles for independent microdo-
 mains of \([\text{Ca}^{2+}]\), on \([\text{Ca}^{2+}]\)-sensitive potassium channels in
SON neurons, and Li and Hatton (20) demonstrated that
internal \([\text{Ca}^{2+}]\) release induced by \([\text{Ca}^{2+}]\) influx is essential for
generation of the phasic pattern of electrical activity, which
most efficiently drives VP release (2). In a similar fashion,
the location of the kinases and transcription factors that are the
postulated triggers for the synergistic hormone release induced
by ATP+PE relative to the sources of increases in \([\text{Ca}^{2+}]\),
(e.g., P2X receptors, endoplasmic reticulum, and voltage-gated
\([\text{Ca}^{2+}]\) channels) will determine the relative influence of each
respective \([\text{Ca}^{2+}]\) source on ATP+PE-induced synergistic re-
lease of VP.

ACKNOWLEDGMENTS

This study was supported by National Institutes of Health Grant RO1-NS27975 to C. Sladek. We thank Adam Zweifach for his help in developing these techniques.

REFERENCES

1. Armstrong WE, Gallagher MJ, and Sladek CD. Noradrenergic stimu-
lation of supraoptic neuronal activity and vasopressin release in vitro:
2. Bicknell RJ. Optimizing release from peptide hormone secretory nerve
3. Buller KM, Khanna S, Sibbald JR, and Day TA. Central noradrener-
gic neurons signal via ATP to elicit vasopressin response to haemorrhage.
4. Buller KM, Smith DW, and Day TA. Differential recruitment of hypo-
thalamic neuroendocrine and ventrolateral medulla catecholamine cells by
non-hypotensive and hypotensive hemorrhages. Brain Res 834: 42–54,
1999.
5. Day TA, Renaud LP, and Sibbald JR. Excitation of supraoptic vaso-
pressin cells by stimulation of the A1 noradrenaline cell group: Failure to
demonstrate role for established adrenergic or amino acid receptors. Brain
6. Day TA, Sibbald JR, and Khanna S. ATP mediates an excitatory noradrenergic
7. Day TA, Sibbald JR, and Smith DW. A1 neurons and excitatory amino
acid receptors in rat caudal medulla mediate vagal excitation of supraoptic
8. Dayanithi G, Widmer H, and Richard P. Vasopressin-induced intracel-

cular \([\text{Ca}^{2+}]\) increase in isolated rat suprapiptic cells. J Physiol 490: 713–
727, 1996.
10. Fisher TE and Bourque CW. Voltage-gated calcium currents in the
magnocellular neurosecretory cells of the rat supraoptic nucleus. J Physiol
11. Foehring RC and Armstrong WE. Pharmacological dissection of high-
voltage-activated \([\text{Ca}^{2+}]\) current types in acutely dissociated rat supraoptic
12. Gieroba ZJ, Spavahl LN, and Blessing WW. Inhibition of the A1 area
prevents hemorrhage-induced secretion of vasopressin in rats. Brain Res
13. Gouzenes L, Sabatier N, Richard P, Moos FC, and Dayanithi G. \(V_{1x}\)
and \(V_{2}\)-type vasopressin receptors mediate vasopressin-induced \([\text{Ca}^{2+}]\)
responses in isolated rat suprapiptic neurons. J Physiol 517: 771–779,
1999.
indicators with greatly improved fluorescence properties. J Biol Chem
15. Hattori Y, Shibuya I, Tanaka K, Kabashima N, Ueta Y, and Ya-
mashita H. Ionotropic and metabotropic glutamate receptor agonist-
induced \([\text{Ca}^{2+}]\), increase in isolated rat suprapiptic neurons. J Neuroen-
16. Hiruma H and Bourque CW. \(P_{2}\) purinoceptor-mediated depolarization
of rat supraoptic neurosecretory cells in vitro. J Physiol 489: 805–811,
1995.
17. Kapoor JR and Sladek CD. Purinergic and adrenergic agonists synergize
in stimulating vasopressin and oxytocin release. J Neurosci 20: 8868–
18. Lambert RC, Dayanithi G, Moos FC, and Richard P. A rise in the
intracellular \([\text{Ca}^{2+}]\) concentration of isolated rat suprapiptic cells in response
19. Li Z. and Hatton GI. Oscillatory bursting of phasically firing rat suprapiptic
neurons in low-\([\text{Ca}^{2+}]/\text{Mg}^{2+}\) medium: \(\text{Na}^{+}\) influx, cytosolic \([\text{Ca}^{2+}]\), and gap
20. Li Z and Hatton GI. \([\text{Ca}^{2+}]\) release from internal stores: role in generating
WE. AHP’s, HAP’s, and DAP’s: How potassium currents regulate the
excitability of rat supraoptic neurons. J Comput Neurosci 15: 367–389,
2003.
22. Sabatier N, Richard P, and Dayanithi G. L- and T- but neither P-
nor \(Q\)-type \([\text{Ca}^{2+}]\) channels control vasopressin-induced \([\text{Ca}^{2+}]\)
 influx in magnocellular vasopressin neurons isolated from the rat supraoptic


