Neurohormone Secretion Persists after Post-Afterdischarge Membrane Depolarization and Cytosolic Calcium Elevation in Peptidergic Neurons in Intact Nervous Tissue

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The purpose of this work was to test the hypothesis that an electrical afterdischarge (AD) causes prolonged elevation in cytosolic calcium levels that is associated with prolonged secretion of egg-laying hormone (ELH) from peptidergic neurons in intact nervous tissue of Aplysia. Using a combination of radioimmunoassay measurement of ELH secretion, electrophysiological measurement of membrane potential, and optical imaging of the concentration of intracellular free calcium ions ([Ca$^{2+}$]), we verified that there was persistent secretion of ELH after the end of the AD; this was accompanied by prolonged post-AD membrane depolarization and prolonged post-AD elevation in [Ca$^{2+}$]. Extracellular treatment with the calcium chelator EGTA had no effect on the pattern or magnitude of ELH secretion or on the post-AD membrane potential ($V_m$) and post-AD Ca$^{2+}$ signal, ruling out a role for extracellular calcium in the post-AD elevation of [Ca$^{2+}$]. Both $V_m$ and [Ca$^{2+}$] returned to baseline well before ELH secretion, such that neither prolonged membrane depolarization nor prolonged Ca$^{2+}$ signaling can fully account for the extent of the persistent secretion of ELH. These findings suggest a unique relationship between membrane excitability, Ca$^{2+}$ signaling, and prolonged neuropeptide secretion.

Key words: action potential; Aplysia; bag cell neurons; calcium imaging; calcium signaling; egg-laying hormone; exocytosis; membrane potential; neuroendocrine; neurosecretion

Dependence of exocytosis on Ca$^{2+}$ influx from extracellular fluid is well documented in a variety of neurons, including dorsal root ganglion cells (Dunlap et al., 1989), motor neurons (Katz and Miledi, 1967), and hypothalamic magnocellular neurons (Mason et al., 1992). These studies and other work have provided a large body of evidence supporting a widely accepted model for the control of neurosecretion that involves membrane depolarization causing opening of voltage-sensitive calcium channels (VSCCs), allowing Ca$^{2+}$ influx, leading to a rise in [Ca$^{2+}$], that is critical for secretion. If Ca$^{2+}$ influx is prevented, then neurosecretion comes to a rapid halt. However, there are several examples of excitable cells and neurons in which secretion is not completely dependent on Ca$^{2+}$ influx. Exocytosis from rat gonadotropes (Tse et al., 1993), GABA secretion from catfish retinal neurons (Schwartz, 1987), and hormone secretion from Aplysia bag cell neurons (BCNs) (Wayne et al., 1998a) have been shown to persist in the absence of Ca$^{2+}$ influx. In both gonadotropes and BCNs, Ca$^{2+}$ release from intracellular stores was sufficient to stimulate secretion (Tse et al., 1997; Wayne et al., 1998a). The present work explored the relationship between membrane excitability, Ca$^{2+}$ signaling, and neuropeptide secretion using the BCNs of the marine mollusk Aplysia as a model system.

Unlike most neurosecretory cells from vertebrate CNS, the BCNs are a homogeneous population of neurons located in two discrete clusters in the Aplysia CNS, facilitating their identification in living tissue. In response to synaptic input, BCNs show a repetitive pattern of synchronous action-potential firing called an afterdischarge (AD) (Kupfermann and Kandel, 1970). This AD triggers exocytotic release of the peptide egg-laying hormone (ELH) (Chiu et al., 1979; Newcomb and Scheller, 1990; Wayne and Wong, 1994). ELH diffuses to target sites at the ovotestis, stimulating ovulation, and at the CNS, altering behaviors associated with egg laying (Rothman et al., 1983; Bernheim and Mayeri, 1995). Work using either intact nervous tissue maintained in vitro or freely behaving Aplysia showed that ELH secretion persisted for $\geq 40$ min after the end of the AD (Wayne, 1994; Wayne and Wong, 1994). Because the BCN Ca$^{2+}$ current is activated at high voltage ($V_m$ more positive than $-10$ mV) (Fieber, 1995), as occurs only during action potential firing, it was hypothesized that extracellular Ca$^{2+}$ would not play an important role in maintaining ELH secretion after the end of the AD. That turned out to be the case. Once the AD was initiated, preventing Ca$^{2+}$ influx did not inhibit ELH secretion (Wayne and Frumovitz, 1995; Wayne et al., 1998a). Furthermore, in the absence of an AD, release of Ca$^{2+}$ from organelles while Ca$^{2+}$ influx from extracellular fluid was blocked was sufficient to stimulate persistent secretion of ELH (Wayne et al., 1998a). The purpose of the present set of experiments was to determine whether AD produces a prolonged Ca$^{2+}$ signal that persists in the absence of Ca$^{2+}$ influx from extracellular fluid and that correlates with the duration of ELH secretion.

Parts of this work have been published previously in abstract form (Wayne and Michel, 2001).

MATERIALS AND METHODS

Animals and solutions. Aplysia californica, weighing 200–300 gm, were purchased from Alacrity Marine Biological Services (Redondo Beach, CA) and maintained in a recirculating seawater system. Water temperature was 20 ± 1°C; a 12 hr light/dark cycle was used. Only animals that...
were reproductively mature and demonstrated the ability to lay eggs in response to injection with an ELH-like peptide from atrial gland extract were used in these experiments (Heller et al., 1980). Before dissection, animals were immobilized by injection of a volume of cold isotonic MgCl2 sufficient to immobilize 30% of their body weight.

Bag cell preparations were maintained and treated with the following solutions. Unless otherwise noted, chemicals were purchased from Sigma (St. Louis, MO). The pH of all external solutions ranged from 7.65 to 7.80; the pH of solutions used in the intracellular recording and microinjection electrode was 7.4. Filtered artificial sea water (ASW) contained the following: 395 mM NaCl, 10 mM KCl, 10 mM CaCl2, 50 mM MgCl2, 28 mM NaH2PO4, 30 mM HEPES, and 5000 U/1 penicillin—streptomycin. EGTA-ASW contained the following (in mM): 419 NaCl, 10 KCl, 5 CaCl2, 50 MgCl2, 28 NaH2PO4, 30 HEPES, and 10 EGTA. The micro-electrode solution contained 0.5 M KCl and 10 mM HEPES. Fura-PE3 radiolabeled ELH within a 5 min sample period (Wayne and Frumovitz, 1996). Animals were immobilized by injection of a volume of cold isotonic chicken egg white, and type III-o ovomucoid trypsin inhibitor from Glendale, CA) and a cocktail of peptidase inhibitors (25 mg/100 ml each of benzamidine, N-ethylmaleimide, benzyl imidazole, leupeptin, aprotinin, leupeptin, pepstatin, antipain, and aprotinin; Sigma, St. Louis, MO). The pH of all external solutions ranged from 7.65 to 7.80.

Microinjection of the calcium indicator dye fura-PE3 was aided by a micromanipulator (Narishige; World Precision Instruments) equipped with a 15° water-immersion objective (0.5 numerical aperture; Olympus Optical, Tokyo). Infrared differential-contrast optics and an infrared camera (OL-1500; Olympus Optical) allowed for the visual selection of BCNs and control of microelectrode impalement and microinjection of dye. BCNs were imaged using a cooled charge-coupled device camera (Sensicam; PCO Computer Optics, Kelheim, Germany) controlled by a personal computer-based imaging and analysis software (Slide-Book; Intelligent Imaging Instruments, Denver, CO). The fluorescence of fura-PE3 was excited alternatively at wavelengths of 340 nm (F340) and 380 nm (F380) using a rotating filter wheel (Lambda 10–2; Sutter Instruments). Emitted light was collected through a dichroic filter, and optical images (12 bits) were acquired every 1–30 sec depending on the experiment. The timing of the optical images was coordinated with the electrophysiology data via a Transistor-Transistor Logic pulses that were triggered by the imaging software and sent to the electrophysiological data acquisition system, in which they were incorporated into the computer-based chart recording. Cells were filled with indicator dye until the fluorescence intensity measured at 340 nm over 20 msec reached 2500 arbitrary units, which provides a sufficient signal without overloading the cells. Calibration was performed in vitro using Ca2+ and EGTA-Ca2+ buffers adjusted for marine ionic composition (in mM: 500 KCl, 50 MgCl2, 10 morpholino-nopropanesulfonate, pH 7.4) and contained between 0 and 30 μM free Ca2+ (calculated using the MAXC program) (Bers et al., 1994). Under these conditions, we determined a Kβ of 1036 nm, and the concentration of free Ca2+ was calculated using the following equation: [Ca2+]free = Kβ × [fura-PE3]0 × (Rmax – (R/R0)) / (Rmax – 1). (Grynkwicz et al., 1985), with R0 = 0.182, Rmax = 2.722, and β = 0.207.

Radioimmunoassay. Concentrations of ELH in ASW were measured using the radioimmunoassay procedure described by Wayne and Wong (1994). For the 11 assays performed, the limit of detection was 1.5 ± 0.2 ng/ml (342 pm; 2 SDs from buffer control values of 100 μl aliquots). The intra-assay coefficient of variation of quadruplicate samples containing 14 ± 0.7 and 34 ± 3 ng/ml averaged 18%, and the interassay coefficient of variation of these samples averaged 20%.

Data analysis. The values in the figures and text are shown as the mean ± SEM. Baseline values for ELH and Vra were defined as the mean ± 2 SDs of those values before electrical stimulation. Calculation of baseline for [Ca2+]i, took into consideration the slope in resting levels, which occurred over the course of 90 min independent of the stimulation of an AD (see Fig. 1). The IGOR data analysis program (WaveMetrics, Lake Oswego, OR) was used to subtract data values of a linear-fitted curve (from beginning to end of recording) from ratiometric values. These “baseline” subtracted values were averaged for all data points before electrical stimulation and 10 min before the end of experiment; 2 SDs above this mean value was considered above baseline. The Pearson correlation test was used to compare the duration of the AD, the decline of post-AD Vra and Ca2+ to baseline, and the total amount of ELH secreted over the entire data set. ANOVA followed by Student’s t test was used to compare the time for [Ca2+]i to decline to baseline in the cell treated normally versus the normal ASW versus EGTA. Student’s t test was used to compare ELH values between normal ASW- and EGTA-treated preparations. Values were considered significantly different at a value of p < 0.05.

RESULTS

Relationship between action potential firing and [Ca2+]i

Figure 1 shows Ca2+ levels under control conditions in which optical images were taken once every 15 sec in the absence of electrical stimulation. There was a consistent slow and progressive increase in resting Ca2+ values after ~40 min. This same increase in the apparent baseline was seen in the majority of our experiments in which afterdischarges were stimulated, even in those studies in which optical images were taken every 30 sec. We cannot account for this change in baseline. Figure 2 illustrates the relationship between action potential firing and the Ca2+ signal during the AD. This example was from a BCN preparation that showed a bursting AD, which is quite rare. Each burst of action potentials was closely followed by a transient increase in [Ca2+]i, as seen in the ratiometric measurement.
Importantly, the fluorescence intensity measurement at 340 nm was the inverse of that at 380 nm during the AD. To simplify the graphics, only the ratiometric data (and derived estimated calcium concentrations) will be shown in subsequent figures.

**Figure 1.** Resting Ca$^{2+}$ levels in BCN soma in the absence of electrical stimulation. Optical images were taken every 15 sec for 90 min from six BCNs from six preparations. The y-axis at left shows data as the ratio of mean background-subtracted fluorescence intensity at 340 and 380 nm wavelength of light. The y-axis at right shows estimated concentrations of cytosolic calcium ([Ca$^{2+}$]$_i$).

**Figure 2.** Ca$^{2+}$ signal during a bursting AD. Top, Images of a single BCN over time; changes in Ca$^{2+}$ levels are shown in pseudocolor, with warmer colors indicating a higher [Ca$^{2+}$]$_i$. Middle, Ca$^{2+}$ signal as the fluorescence intensity at 340 nm ($F_{340}$) and 380 nm ($F_{380}$) wavelength of light and as the ratio of $F_{340}$ to $F_{380}$. Bottom, Changes in $V_m$ and action potential firing during the bursting AD. Scale bars for fluorescence intensity (in arbitrary units) and for the ratio of $F_{340}/F_{380}$ are shown at right. Time axis is the same for all panels.

**Figure 3.** Relationship between the AD (A; shown as the number of action potentials per minute), $V_m$ (B; shown in millivolts; interspike interval during the AD), Ca$^{2+}$ concentration (C; shown as both the ratio $F_{340}/F_{380}$ and estimated [Ca$^{2+}$]$_i$), and ELH secretion (D; in nanograms per milliliter) from BCN soma in preparations maintained in normal ASW. B–D, Shaded areas represent the timing and duration of the averaged AD. B, C, Dashed lines indicate resting $V_m$ and Ca$^{2+}$ levels, respectively. B shows $V_m$ recordings from five of the eight preparations in this experiment (the electrode came out of the cell from the remaining 3 preparations early in the experiment).

### Relationship between membrane excitability, [Ca$^{2+}$]$_i$, in the soma, and ELH secretion

Figure 3 shows the pattern of action potential firing (A), $V_m$ (interspike $V_m$ shown during the AD), [Ca$^{2+}$]$_i$, and ELH secretion before, during, and after the end of the AD. The average duration of the AD was 11 ± 2 min. Notably, $V_m$, [Ca$^{2+}$]$_i$, and ELH secretion all persisted after the end of the AD in eight of eight preparations (Table 1). ELH levels were still above baseline in seven of eight preparations by the end of the 90 min experiment. Taking into account the 10 min delay in clearance of ELH from the recording chamber, all eight preparations were still secreting ELH after $V_m$, and [Ca$^{2+}$]$_i$ declined to baseline.
Effect of preventing Ca$^{2+}$ influx on membrane excitability, [Ca$^{2+}$], in the soma, and ELH secretion

Initiation and maintenance of the AD requires Ca$^{2+}$ influx, and, in turn, activation of ELH secretion is dependent on the AD (Wayne and Frumovitz, 1995). To test whether Ca$^{2+}$ influx plays an important role in post-AD BCN functions, it was necessary to first initiate the AD and then prevent Ca$^{2+}$ entry. In the present experiment, treatment with the calcium chelator EGTA was initiated within 30 sec of the onset of the AD. This solution contains 39 nM free Ca$^{2+}$ (Wayne and Frumovitz, 1995), which is below resting [Ca$^{2+}$]$_{i}$. The reduction of extracellular [Ca$^{2+}$] led to a shortened AD, averaging $5 \pm 1$ min (range, 7 sec to 9 min), compared with preparations stimulated in normal ASW ($p < 0.005$) (compare Figs. 3, 4). The variability in the duration of AD of the EGTA-treated group can be accounted for by inconsistencies in the rate at which individual preparations achieved complete perfusions of solution, which is partly a function of variability in vascularization of the abdominal ganglia. Figure 4 and Table 1 show that, compared with preparations stimulated in the presence of normal ASW, treatment with EGTA had no effect on the post-AD decline in $V_m$ or [Ca$^{2+}$]. Furthermore, as shown previously (Wayne and Frumovitz, 1995; Wayne et al., 1998a), preventing Ca$^{2+}$ influx did not inhibit ELH secretion (Table 1). ELH had not declined to baseline by the end of the experiment in any of the preparations in this group. Even the preparation that showed a 7 sec AD (just 43 action potentials) secreted high amounts of ELH over a prolonged period of time (total of 683 ng/ml for $\geq 90$ min). This result indicates that the post-AD elevation in [Ca$^{2+}$]$_{i}$ is not a result of Ca$^{2+}$ influx from extracellular fluid.

Ca$^{2+}$ signal in the neurite

Figure 5 shows the relationship between membrane excitability in BCNs and [Ca$^{2+}$]$_{i}$ in their respective neurites, in which presumably most of ELH secretion is taking place (Fisher et al., 1988; Roubos et al., 1990). BCN neurites vary tremendously in length, from $< 500 \mu \text{m}$ for those that terminate within the BCN cluster to several centimeters for those that traverse the pleurovisceral connective nerve toward the head ganglia (Kaczmarek et al., 1979; Shope et al., 1991). The calcium indicator dye, however, did not diffuse in sufficient quantities to monitor fluorescence past 120 $\mu \text{m}$ along the neurites with the optics used in this study. Therefore, all neurite data are shown in a region that is within 100 $\mu \text{m}$ of the soma. As in the soma, there was a slow decline in the levels of Ca$^{2+}$ to baseline in the neurite; however, [Ca$^{2+}$]$_{i}$ in the neurite reached baseline significantly earlier than that in the soma (Table 1). Figure 6 shows an example of a BCN in which the Ca$^{2+}$ signal was monitored in the soma, the proximal neurite, and what is most likely the neurite terminal. The fluorescence in this BCN ended abruptly in a knob-like structure, with no out-of-plane fluorescence in that region, thus suggesting that the knob was the terminal of a short neurite (as described by Kaczmarek et al., 1979). Although there were slight differences in the magnitude of Ca$^{2+}$ levels in those three areas of the neuron during the AD, in this example, all three regions showed a similar slow post-AD decline in Ca$^{2+}$ toward baseline. Overall, these findings indicate that the Ca$^{2+}$ signal remains elevated above baseline after the end of the AD in all regions of the BCN, at least when we
time that [Ca$^{2+}$]$_i$ declined to baseline ($r = -0.22$), or the total amount of ELH secreted ($r = -0.14$). There was no significant correlation between total amount of ELH secreted and the time that $V_m$ declined to baseline ($r = 0.12$) or the time that [Ca$^{2+}$]$_i$ declined to baseline ($r = 0.26$). These findings suggest that the AD acts as a triggering mechanism rather than a sustained driving force for downstream cellular events, including prolonged membrane depolarization, Ca$^{2+}$ release from intracellular stores, and ELH secretion. Furthermore, neither prolonged membrane depolarization nor post-AD elevation in [Ca$^{2+}$]$_i$ can account for the entire post-AD period of ELH secretion.

**DISCUSSION**

Influx of Ca$^{2+}$ from extracellular fluid and elevated [Ca$^{2+}$]$_i$, are commonly associated with secretion of transmitter or peptide from neurons (Bennett, 1997; Kits and Mansvelder, 2000). Our previous work brought into question whether this model could be extended to BCNs that secrete peptide hormone over a prolonged period of time. Preventing Ca$^{2+}$ influx shortly after onset of the AD did not inhibit ELH secretion, although the duration of the AD was significantly abbreviated (Wayne and Fromowitz, 1995; Wayne et al., 1998a) (Fig. 4). Furthermore, release of Ca$^{2+}$ from intracellular stores in the absence of both AD and Ca$^{2+}$ influx stimulated sustained ELH secretion (Wayne et al., 1998a). These findings in BCNs suggested a model in which the AD triggers some cellular event(s) leading to prolonged release of Ca$^{2+}$ from intracellular stores that might drive prolonged ELH secretion. Our present findings confirm that, once the AD is initiated, Ca$^{2+}$ influx from extracellular fluid is not necessary to maintain a normal pattern of ELH secretion. The results also show that not only does the AD trigger prolong ELH secretion, but also leads to prolonged membrane depolarization and Ca$^{2+}$ elevation, all of which persist after the end of the AD. This post-AD elevation in [Ca$^{2+}$]$_i$ was not altered in the presence of extracellular EGTA, indicating that it was not a result of Ca$^{2+}$ influx from extracellular fluid. Notably, $V_m$ repolarized to resting levels and [Ca$^{2+}$]$_i$ declined to baseline well before ELH secretion. Therefore, the post-AD membrane depolarization and elevation in [Ca$^{2+}$]$_i$ cannot fully account for the prolonged release of ELH; perhaps some additional cellular event is playing an important role in mediating the effect of AD on neurohormone secretion. Importantly, this work was done using an intact nervous preparation in which the integrity of the neuroendocrine network was maintained in vitro. Changes in membrane excitability and hormone secretion from this excised BCN preparation are comparable with those observed in freely behaving animals, suggesting that the data from the present study are of physiological and behavioral relevance (Wayne, 1994, 1995).

There is precedence in other neurosecretory cells for persistent elevation in [Ca$^{2+}$]$_i$ after the end of some stimulus. Previous work in reproductive neuroendocrine cells in the freshwater pond snail *Lymnaea stagnalis* that are highly analogous to BCNs showed that [Ca$^{2+}$]$_i$ in the peptidergic caudodorsal cell neurons rose threefold to fourfold during an electrical AD and, as with BCNs, remained elevated for tens of minutes after the end of the AD (Kits et al., 1997). However, simultaneous measurements of caudodorsal cell hormone secretion and Ca$^{2+}$ have not been reported; thus, the temporal relationship between the Ca$^{2+}$ signal and caudodorsal cell hormone has not been determined. The gonadotropin releasing hormone (GnRH) neurons of the mammalian hypothalamus and the gonadotropes of the anterior pituitary provide another example of a reproductive system in which

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**Evaluation of possible correlations between different BCN functions**

There was no significant correlation between the duration of the AD and the time that $V_m$ declined to baseline ($r = -0.23$), the
Ca\textsuperscript{2+} release from intracellular stores plays an important role in regulating peptide hormone release. In this mammalian system, GnRH binds to receptors on pituitary gonadotropes, activating Ca\textsuperscript{2+} oscillations that are accompanied by rhythmic exocytosis. Both the Ca\textsuperscript{2+} oscillations and rhythmic exocytosis persist after the end of the GnRH stimulus, occur in the absence of Ca\textsuperscript{2+} influx from extracellular fluid, and can be triggered by an IP\textsubscript{3}-mediated mechanism that releases Ca\textsuperscript{2+} from intracellular stores (Tse et al., 1993). Work in embryonic primate GnRH neurons has shown that, as with the gonadotropes, there are oscillations (Terasawa et al., 1999a,b). Although preventing Ca\textsuperscript{2+} influx eliminated pulsatile GnRH secretion, there was evidence that Ca\textsuperscript{2+} release from intracellular stores contributed to some aspect of the pattern of GnRH secretion. That is, pharmacological mobilization of Ca\textsuperscript{2+} from ryanodine-sensitive and mitochondrial stores stimulated significant amounts of GnRH secretion (Terasawa et al., 1999a).

In the present study, the post-AD elevation in [Ca\textsuperscript{2+}], persisted in the presence of extracellular EGTA. This slow decline of [Ca\textsuperscript{2+}], could be a result of inefficient buffering or extrusion of Ca\textsuperscript{2+} after the end of the AD. The kinetics of Ca\textsuperscript{2+} buffering in other cell types is reported to be on the order of seconds (Xu et al., 1997; Kits and Mansvelder, 2000) and not tens of minutes; however, this process could be extremely slow in BCNs. Another possible explanation for the post-AD elevation in [Ca\textsuperscript{2+}], is release of Ca\textsuperscript{2+} from intracellular stores. There is evidence for this in BCNs from previous studies showing that IP\textsubscript{3} stimulates an increase in [Ca\textsuperscript{2+}], in both soma and neurites (Fink et al., 1988) and that Ba\textsuperscript{2+} influx through VSCCs can trigger Ca\textsuperscript{2+} release as measured by a calcium-selective electrode in the cell soma (Fisher et al., 1994). This brings up the possibility that, during the AD, multiple pathways could be activated that contribute to the Ca\textsuperscript{2+} signal, including Ca\textsuperscript{2+} influx from extracellular fluid, Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release from intracellular stores, and synaptic stimulation of an IP\textsubscript{3}-sensitive store. The BCN intracellular store that might be contributing to the AD-induced Ca\textsuperscript{2+} signal has not yet been identified.

There is a temporal dissociation between peak [Ca\textsuperscript{2+}], and peak ELH secretion in the present study. Quite noticeably, the bulk of the Ca\textsuperscript{2+} signal occurred during the AD, whereas the bulk of ELH secretion occurred after the end of the AD. This delay in ELH secretion relative to the pattern of action-potential firing and [Ca\textsuperscript{2+}], cannot be accounted for by the delay in clearance of solution through the recording chamber, because there is 95% clearance within two samples or 10 min. Importantly, in freely behaving Aplysia, concentrations of ELH in hemolymph are elevated for 40 min after the end of the AD, although this hormone has a half-life of ~3 min (Wayne, 1994). Also, in the present study, [Ca\textsuperscript{2+}], reached baseline well before ELH secretion declined to baseline, suggesting that maintenance of prolonged ELH secretion is not dependent on either Ca\textsuperscript{2+} influx from extracellular fluid or release from intracellular stores. If Ca\textsuperscript{2+} is not driving prolonged ELH secretion, then what alternative mechanisms could be stimulating peptide release?

One potential component is membrane depolarization leading to some long-lasting Ca\textsuperscript{2+}-independent mechanism. Certainly, the AD led to a robust and sustained depolarization of V\textsubscript{m}, that was very slow to repolarize to resting levels. However, like the Ca\textsuperscript{2+} signal, V\textsubscript{m} reached baseline well before ELH secretion. Nevertheless, prolonged membrane depolarization could be stimulating an even longer activation of some other signaling pathway that plays an important role in ELH secretion.

Our previous work showed that both cAMP-dependent protein kinase and calcium/phospholipid-dependent protein kinase (PKC) play important roles in mediating the effects of AD on ELH secretion (Wayne et al., 1998b). Furthermore, AD stimulated rapid and prolonged activation of both the calcium-activated and calcium-independent forms of PKC from BCNs, with a time course that was similar to that of ELH secretion (Wayne et al., 1999). Given the findings in the present study, it is especially intriguing that the calcium-independent PKC showed persistent activation in response to AD. Work in rat anterior pituitary gonadotropes has also implied a role for PKC in stimulating peptide hormone secretion in the absence of elevated [Ca\textsuperscript{2+}]. (Billiard et al., 1997). Additional support for Ca\textsuperscript{2+}-independent exocytosis in neurons comes from studies in which ethanol-induced secretion in avian ciliary ganglion neurons (Broius et al., 1992), nitric-oxide induced exocytosis in hippocampal syncytomes (Meffert et al., 1994), and Na\textsuperscript{+}-induced secretion in neurohypophysial nerve endings (Stuenkel and Nordmann, 1993) occurred in the absence of increases in [Ca\textsuperscript{2+}]. Although the biochemical–molecular mechanism(s) by which exocytosis can be activated in the absence of a rise in [Ca\textsuperscript{2+}], has not been revealed, the possibility of kinase- or neurmodulator-activated allosteric modification of Ca\textsuperscript{2+}-sensitive exocytotic proteins has been postulated as a potential mechanism (Meffert et al., 1994; Billiard et al., 1997). Our findings in BCNs lend support to the importance of an alternative mechanism controlling neurosecretion in which neither Ca\textsuperscript{2+} influx nor elevated [Ca\textsuperscript{2+}], is required.

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