J. Membrane Biol. 128, 41–52 (1992)

The Journal of
Membrane Biology

Hyperosmotic Media Inhibit Voltage-Dependent Calcium Influx and Peptide Release in *Aplysia* Neurons

Karen J. Loechner, Ronald J. Knox, John A. Connor,† and Leonard K. Kaczmarek
Department of Pharmacology, Yale University School of Medicine, New Haven, Connecticut, and †Department of Neuroscience, Roche Research Center, Nutley, New Jersey 07110

Summary. The bag cell neurons of *Aplysia* provide a model system in which to investigate the effects of hyperosmolality on the electrical and secretory properties of neurons. Brief stimulation of these neurons triggers an afterdischarge of action potentials that lasts approximately 20–30 min, during which time they release several neuroactive peptides. We have found that pre-incubation of intact clusters of bag cell neurons in hyperosmotic media prior to stimulation prevents the initiation of afterdischarges. Furthermore, an increase in osmolality of the external medium during an ongoing afterdischarge causes its premature termination. Hyperosmotic media attenuate the release of peptide evoked by both electrically stimulated afterdischarges and potassium-induced depolarization. The ability of high potassium to depolarize the bag cell neurons is, however, not impaired. Exposure of isolated bag cell neurons to hyperosmotic media also inhibits the amplitude of action potentials evoked by depolarizing current injection and attenuates the voltage-dependent calcium current. In isolated bag cell neurons loaded with the calcium indicator dye, fura-2, hyperosmotic media reduced the rise in intracellular calcium levels that normally occurs in response to depolarization. Our results suggest that the effects of hyperosmotic media on peptide secretion in bag cell neurons can largely be attributed to their effects on calcium entry.

Key Words osmolality · calcium current · egg-laying hormone · secretion · bag cell neurons

Introduction

Alterations in extracellular osmolality have been shown to affect calcium-dependent exocytosis in a variety of systems. For example, hyperosmotic solutions block the release of granules from adrenal chromaffin cells (Knight & Baker, 1982; Hampton & Holz, 1983; Holz & Senter, 1986; Ladona, Bader & Aunis 1987; O'Sullivan & Burgoyne, 1988), sea urchin eggs (Whitaker & Zimmerberg, 1987; Chandler, Whitaker & Zimmerberg 1989; Merkle & Chandler 1989), neutrophils (Kazilek, Merkle & Chandler, 1988), parathyroid cells (Brown et al., 1978), and GH_4C_1 cells (Sato, Wang & Greer, 1991). It has been proposed that hyperosmotic media directly inhibit granule fusion with the plasma membrane (Chandler et al., 1989; Merkle & Chandler, 1989). It has also been suggested that increasing extracellular osmolality may alter osmotic gradients necessary for swelling of granules and subsequent extrusion of granule contents (Whitaker & Zimmerberg, 1987; Chandler et al., 1989). It is possible, however, that hyperosmotic media influence stages in excitation-secretion coupling that precede the fusion of granules with the plasma membrane. For example, hyperosmotic media have been shown to influence the gating of potassium ion channels (Zimmerberg, Bezanilla & Parsegian, 1990). Hyperosmotic media have also been shown to block potassium- or hormone-induced calcium influx in chromaffin cells (Heldman et al., 1991) and GH_4C_1 cells (Sato et al., 1991). In rabbit neutrophils, hyperosmotic media inhibit the rise in intracellular calcium that normally occurs in response to chemotactic peptide (Kazilek et al., 1988). The mechanisms of inhibition of the rise in calcium levels have, however, not been determined.

We have now investigated the effects of hyperosmotic media on stimulus-secretion coupling in the peptidergic bag cell neurons of the marine mollusc *Aplysia*. Following brief electrical stimulation, these neurons fire repetitively for approximately 20–30 min, during which time they release several neuroactive peptides. These peptides include egg-laying hormone (ELH), a 36 amino acid peptide that, in vivo, induces egg-laying behavior (Arch, 1972; Chiu et al., 1979; Scheller et al., 1982). We have measured voltage-dependent calcium currents in isolated bag cell neurons in primary culture and have used imaging techniques to measure the calcium transients that may be associated with secretion. Our results indicate that hyperosmotic media influence secretion by attenuating calcium entry through voltage-dependent calcium channels.

Table 1. Composition of solutions A–N[a]

	A	B	C	D	E	F	G	H	I	J	K	L	M	N
NaCl	460	460	460	760	460	160	460	160	160	160	—	—	30	30
$MgCl_2$	55	55	55	55	55	55	55	55	55	55	55	55	55	55
KCl	10.4	10.4	10.4	10.4	10.4	310.4	310.4	310.4	310.4	310.4	—	—	—	—
$CaCl_2$	11	11	11	11	11	11	11	11	11	11	—	—	11	11
$BaCl_2$	—	—	—	—	—	—	—	—	—	—	11	11	—	—
CsCl	—	—	—	—	—	—	—	—	—	—	10.4	10.4	10.4	10.4
TEA-Br	—	—	—	—	—	—	—	—	—	—	460	460	460	460
DAP	—	—	—	—	—	—	—	—	—	—	—	—	30	30
EGTA	—	—	—	—	—	—	—	—	—	5	—	—	—	—
HEPES	10	10	10	10	10	10	10	10	10	10	—	—	—	—
Tris	—	—	—	—	—	—	—	—	—	—	10	10	10	10
Mannitol	—	600	—	—	—	—	—	600	—	—	—	600	—	600
Choline-Cl	—	—	300	—	—	—	—	—	300	—	—	—	—	—
Sucrose	—	—	—	—	600	—	—	—	—	—	—	—	—	—

[a] Concentrations listed are in mM. All solutions were adjusted to pH = 7.8.

This work has been presented in abstract form (Loechner & Kaczmarek, 1989; Loechner et al., 1991).

Materials and Methods

Animals

Adult *Aplysia californica* (weight 250–500 g) (Alacrity Marine, Redondo Beach, CA) were kept in an artificial seawater system at 13°C.

Extracellular Recording

Prior to dissection, animals were anesthetized by an injection of $MgCl_2$ (~1200 mosm) equal to 50% of body weight. The abdominal ganglion with bag cell clusters and pleurovisceral connective nerves was then removed and placed in a 1-ml chamber containing a solution made up of equal volumes of artificial seawater (ASW, ~1200 mosm; *see* Solution A, Table 1) and $MgCl_2$. The artery to the abdominal ganglion was cannulated with a plastic syringe and secured as described by Mayeri et al. (1985). $MgCl_2$ was included to prevent the premature stimulation of afterdischarges during dissection and cannulation. The ganglion was then washed with ASW prior to each experiment. Experiments were carried out at 18°C.

An extracellular suction-recording electrode was placed on one bag cell cluster, and an extracellular suction-stimulating electrode placed on the ipsilateral connective nerve. Afterdischarges were stimulated by application of a train of brief depolarizing current pulses (8–40 V, 6 Hz, 2.5 msec pulse width, 5 sec duration). Bag cell neuron electrical activity was monitored on an oscilloscope and recorded on a polygraph chart recorder.

During each experiment, the extracellular medium was completely exchanged at 5 min intervals. The artery was perfused at 9 μl/min with the same solutions as the extracellular medium. Samples were frozen and stored at −20°C until analyzed for peptide composition.

Analysis of Peptides

The separation and detection of peptides were carried out using high pressure liquid chromatography as described by Loechner et al. (1990). Briefly, samples were thawed immediately prior to analysis and aliquots were injected onto a Vydac C18 reverse phase column (5 μM, 300 A, 2.5 × 15 cm). Peptides were separated using a segmental linear gradient of 0.05% trifluoroacetic acid in acetonitrile (5–41% acetonitrile in 39 min at a flow rate of 1 ml/min).

Post-column derivitization with *o*-phthalic dicarboxaldehyde in the presence of 2-mercaptoethanol (Roth, 1971) was carried out and peptides were detected using a Hitachi F-1600 spectrophotometer. ELH was quantified according to peak height on a chart recorder. Peak height was then converted to the corresponding amount of peptide in micrograms based on synthetic ELH standards (Peninsula Laboratories, Belmont, CA). All measurements are expressed as ± SEM.

Manipulation of Osmolality

To analyze the effects of hyperosmotic solutions on ELH secretion and afterdischarges, the extracellular medium and cannula contained ASW prior to stimulation of the bag cell neurons. Following 5 min of afterdischarge, the extracellular and perfusing media were changed to ASW made hyperosmotic (~1800 mosm) with mannitol, choline chloride, or sodium chloride (*see* Table 1, solutions B, C, and D, respectively). Samples were collected at 5-min intervals for at least 25 min following termination of electrical activity.

To analyze the effect of hyperosmotic solutions on the ability of electrical stimulation to trigger an afterdischarge in bag cell neurons, experiments were carried out as above except that both the extracellular and perfusing media were exchanged with solution B (mannitol) or solution C (choline chloride) at least 5 min prior to electrical stimulation.

Measurements of Potassium-Evoked Release

To measure the release of ELH in response to depolarization by elevated potassium concentrations, basal samples of release were first collected using normal ASW for both the extracellular and

perfusing media. The medium was then changed to one of the following four solutions: (i) isosmotic high potassium, in which potassium chloride is partially substituted for sodium chloride in ASW (solution F), (ii) hyperosmotic high potassium (solution G, sodium chloride), which differs from isosmotic high potassium in its sodium chloride concentration (solution G), (iii) hyperosmotic high potassium (solution H, mannitol), or (iv) hyperosmotic high potassium (solution I, choline chloride). Samples were collected at 5 min intervals as described above.

To test the calcium dependence of potassium-evoked release, both the extracellular and perfusing media were changed to a medium containing high potassium in 0 Ca^{2+} ASW to which 5 mM ethylene glycol-*bis*-(β-aminoethyl ether)-N,N,N′N′-tetraacetic acid (EGTA) was added to chelate residual calcium (solution J).

Intracellular Recording

Abdominal ganglia were removed from the animals as described above. The ganglia were then placed in a dish containing 15 mg/ml Dispase (Boehringer Mannheim, Germany) for at least 18 hr. The two bag cell clusters were then dissected from the ganglion and individual neurons separated by trituration of the clusters using a Pasteur pipette. Cells were plated onto petri dishes (Corning) containing ASW (to which glucose (1 mg/ml), penicillin and streptomycin (Gibco) were added) and stored in an incubator at 15°C. Isolated cells were used for experiments within two days following plating. To record action potentials, isolated bag cell neurons in primary culture were penetrated with a microelectrode containing 3 M potassium chloride (pH = 7.3, resistance = 10–40 MΩ in ASW). Action potentials were stimulated by injection of depolarizing current (50–500 pA, 250 msec pulse) before, during, and after exchange of ASW with hyperosmotic ASW (solution B, mannitol; solution C, choline chloride; or solution E, sucrose). Action potentials were measured at peak height and changes expressed as percentage ± SEM. To depolarize the cells with elevated potassium concentration, 100 μl of 3 M potassium chloride was added to dishes containing 3 ml of either isosmotic ASW or hyperosmotic ASW (solution B, mannitol (final concentration of potassium chloride in dish = 100 mM).

To measure voltage-dependent calcium currents, isolated bag cell neurons were washed with solutions containing either barium (solution K) or calcium (solution M) as charge carriers, as well as tetra-ethylammonium bromide and cesium chloride to block outward potassium currents (*see* Table 1). These solutions allow inward currents to be measured relatively uncontaminated by the outward potassium currents. Neurons were penetrated with a single microelectrode containing 3 M potassium chloride (pH = 7.3, resistance = 8–15 MΩ). Calcium current was measured using a single microelectrode voltage clamp (Axoclamp). The membrane potential was held at −60 mV and then depolarized to test potentials between −20 to +10 mV (250 msec pulse, interval between pulses = 30 sec). Inward current was monitored before and after exchange of isosmotic solutions K (barium) and M (calcium) with barium-containing hyperosmotic solution L (mannitol) or calcium-containing hyperosmotic solution N (mannitol), and following return to the respective isosmotic media. Measurements were made at peak inward current and changes are expressed as percentage ± SEM.

To measure leakage currents, cells were depolarized to −50 mV from a holding potential of −60 mV. Leakage current was monitored before and after exchange with hyperosmotic media as described above.

Measurements of Intracellular Calcium

Bag cell neurons were plated on #1 glass microscope coverslips coated with polylysine substrate and allowed to extend neurites for a minimum of 18 hr, at which time they had extended elaborate growth cones characteristic of *Aplysia* and other invertebrate neurons in primary culture (Kaczmarek et al., 1979; Kater et al., 1988; Knox et al., 1992). Fura-2 (free acid) (Grynkiewicz, Poenie & Tsien, 1985) was microinjected into the bag cell soma to concentrations of 50–100 μM by pressure injection from intracellular microelectrodes (electrode resistance >50 MΩ when filled with 3 M KCl). Microinjections took 20–40 sec and cells were allowed to equilibrate for at least 10 min after fura-2 was injected. For electrical stimulation and recording, bag cell neurons were repenetrated with identical microelectrodes filled with 1 M KCl. Quantification of intracellular calcium changes in single cells was determined by taking the ratio of fura-2 fluorescence using 340 and 380 nm excitation wavelengths in pairs of digitized images. Acquisition time for a single frame pair was approximately 500 msec. Free calcium is related to this ratio as follows:

$$[Ca^{2+}] = K_d\left(\frac{R - R_{min}}{R_{max} - R}\right)\left(\frac{F_o}{F_s}\right)$$

where R_{min} (0.45) and R_{max} (11.5) are minimum and maximum ratios observed in the apparatus for the limiting values of calcium and F_o/F_s is the fluorescence ratio for 380 nm excitation at the limiting values of calcium. K_d is the indicator dissociation constant and equals 760 nM (*see* Grynkiewicz et al., 1985). The computed calcium levels are coded in pseudo-color from blue to red, corresponding with low to high levels. Details of the method and imaging apparatus have been decribed previously (Connor, 1986; Fink, Connor & Kaczmarek, 1988). Neurons were allowed to remain in ASW or were washed with hyperosmotic ASW prior to stimulation. Calcium influx was evoked by stimulating action potentials with depolarizing current injection (0.5–1.0 nA, 75 msec duration) or by exchange of the medium to one containing high potassium as described above. Calcium concentrations are expressed as nanomolar ± SEM.

Results

Peptide Release during the Bag Cell Afterdischarge

The bag cell neurons form two clusters of approximately 400 neurons each at the rostral end of the abdominal ganglion of *Aplysia*. (Frazier et al., 1967). Prior to stimulation, these neurons are electrically silent (Fig. 1*A*, *Basal*). Following brief electrical stimulation (*S*) of the pleurovisceral connective nerve, the bag cell neurons fire repetitive action potentials for 20–30 min (Kaczmarek, Jennings & Strumwasser, 1982). Since these neurons are electronically coupled, the compound action potentials recorded extracellularly represent the synchronous activity of all neurons in a cluster (Blankenship & Haskins, 1979; Kaczmarek et al., 1979). As seen in Fig. 1*A*, the pattern of activity changes during the

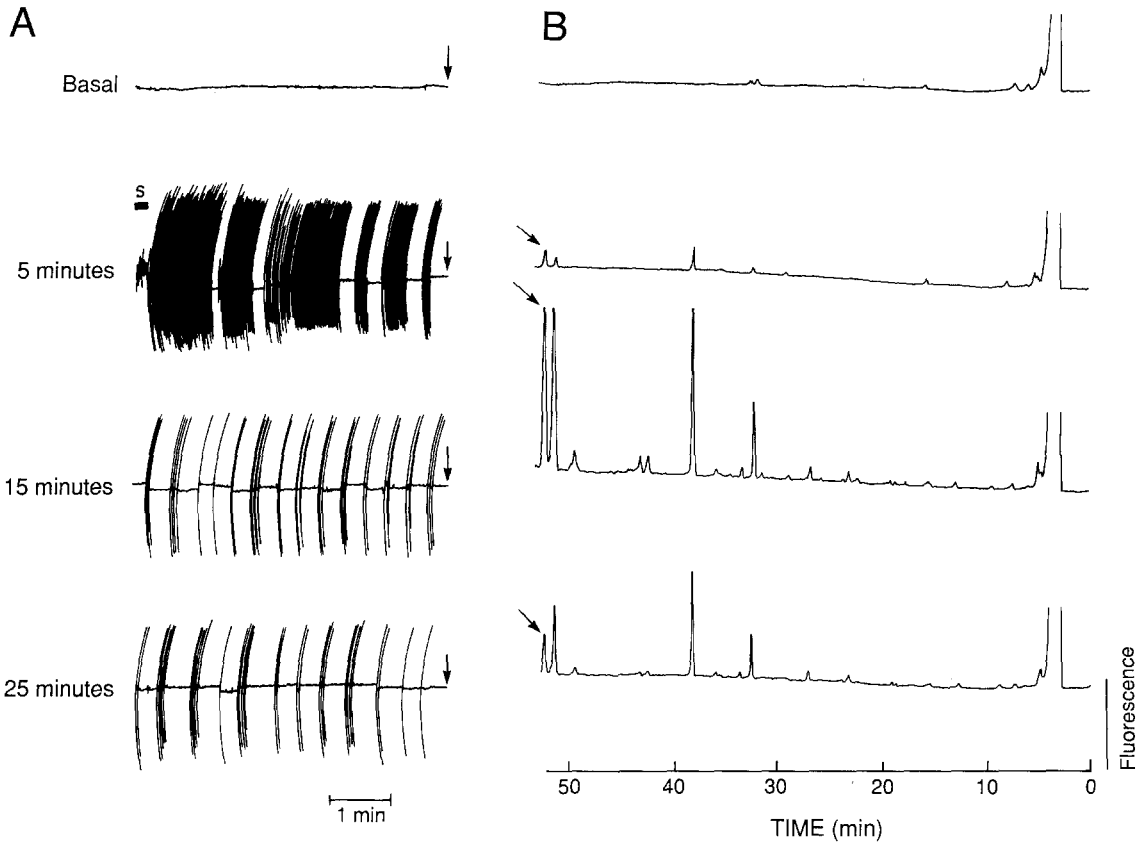

Fig. 1. (*A*) Extracellular recording of bag cell neuron electrical activity before (*Basal* and after stimulation (*S*) of an afterdischarge. Arrows indicate point at which the extracellular medium was exchanged. Alternate 5-min samples are shown for simplicity. (*B*) HPLC chromatograms corresponding to each 5-min period of electrical activity. Arrows designate ELH peaks. *X* axis represents time during HPLC gradient; *Y* axis represents relative fluorescence.

course of the afterdischarge. During the first phase, which lasts about 1 min, the neurons fire at ~5 Hz. During the second, slower phase, which lasts the remainder of the afterdischarge, action potentials occur at ~0.5 Hz (Kaczmarek et al., 1982). Previous work has shown that action potentials during the fast phase are largely sodium-dependent, whereas in the second phase, calcium entry plays an important role in determining the shape and firing pattern of action potentials (Kaczmarek et al., 1982).

Figure 1*B* shows HPLC profiles of peptides released into the extracellular medium during each 5 min of electrical activity. No peptides were detected prior to stimulation (basal). Following initiation of the afterdischarge, however, several peptides could be detected, including ELH (egg-laying hormone) (Rothman, Sigvardt & Mayeri, 1985; Loechner et al., 1990). The pattern of ELH release during the course of the afterdischarge as shown in Fig. 1*B* is typical of that seen in clusters stimulated in ASW, such that a greater amount of ELH is released at intervals subsequent to the first 5 min, even though the period of maximal firing occurs during these first minutes (Loechner et al., 1990).

The bag cell neurons release several other neuropeptides in addition to ELH. Following electrical stimulation or elevated potassium (*see* Fig. 3*A*), several other peaks can be detected on HPLC chromatograms of released material. The peak adjacent to that of ELH represents Acidic Peptide, which is cleaved from the ELH precursor but whose function is not yet known (Scheller et al., 1983; Newcomb & Scheller, 1987). The other non-ELH peaks have not been identified. They do not, however, represent alpha-, beta-, or gamma-bag cell peptides, which are smaller peptides also produced from the ELH precursor protein and released during the afterdischarge (Rothman et al., 1985; Scheller et al., 1983). These peptides, which produce autoreceptor-mediated changes in the bag cell neurons as well as act on target neurons in the abdominal ganglia (Rothman et al., 1983; Sigvardt et al., 1986; Kauer, Fisher & Kaczmarek, 1987; Berry, 1988; Brown & Mayeri, 1989; Loechner & Kaczmarek, 1990), are susceptible to rapid proteolysis and are released in amounts too small to be detected in present experiments. The non-ELH peaks on the chromatograms may represent other products of the ELH precursor

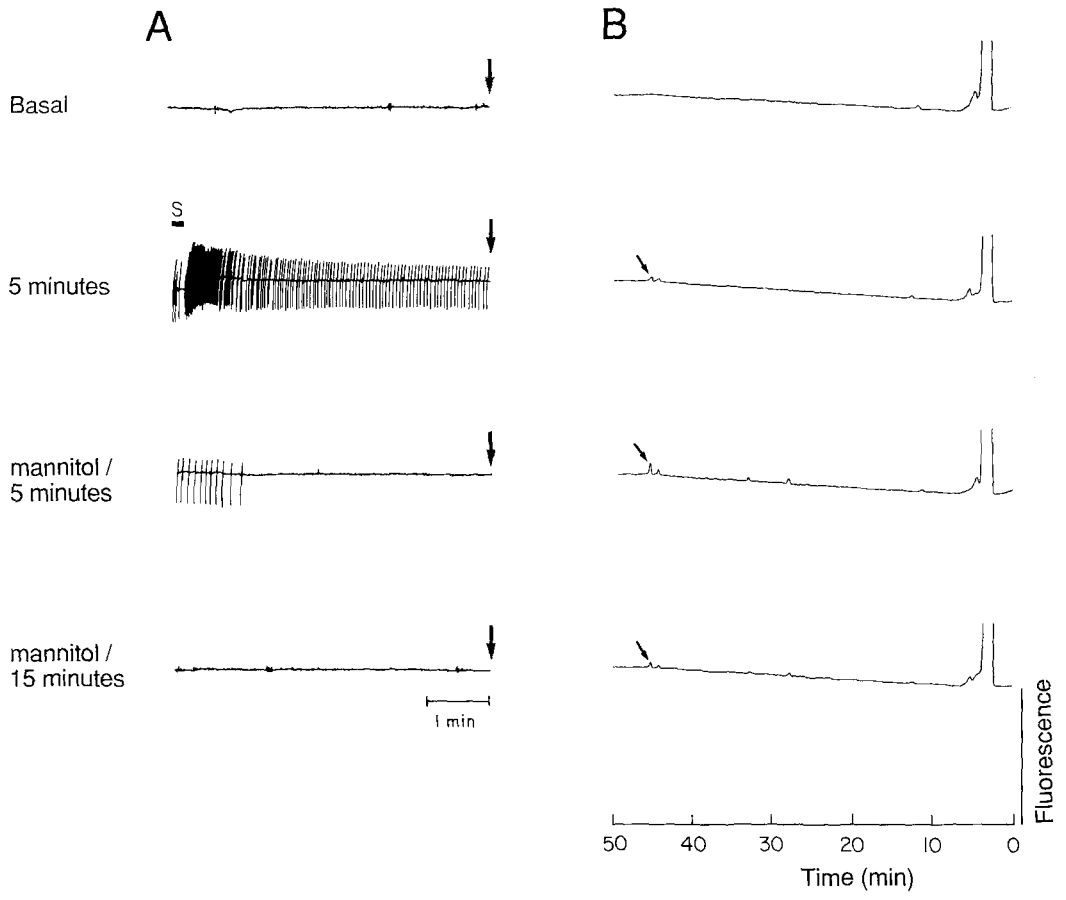

Fig. 2. (*A*) Extracellular recording of bag cell neuron electrical activity before (*Basal*) and after stimulation (*S*) of an afterdischarge. Arrows indicate point at which the extracellular medium is exchanged. Following the first 5 min of action potentials, isosmotic ASW solution A was completely replaced with hyperosmotic ASW solution B (mannitol). (*B*) HPLC profiles corresponding to each 5-min period in *A*. Arrows designate ELH peaks. *X* axis represents time on HPLC gradient; *Y* axis represents relative fluorescence.

or products released from other neurons within the abdominal ganglion.

Inhibition of ELH Secretion and Afterdischarges by Hyperosmotic Media

We examined the effect of hyperosmotic media on the release of ELH that normally occurs during an afterdischarge. After the first 5 min of repetitive firing, both the extracellular and perfusing media were replaced with hyperosmotic ASW solution B (mannitol) (Fig. 2). Within 2 min of exposure to this hyperosmotic medium, bag cell electrical activity ceased (Fig. 2*a*; $n = 4$). This is in contrast to the control clusters, which fired repetitively throughout the afterdischarge (Fig. 1*A*). As can be seen, the amount of ELH detected by HPLC was greatly attenuated following exchange with hyperosmotic ASW (Fig. 2*B*) when compared to that obtained under isosmotic conditions (Fig. 1*B*). The mean release of ELH during the 5-min interval following exchange with hyperosmotic solution B was 0.12 ± 0.02 μg ($n = 4$); ELH release in isosmotic solution A was 1.33 ± 0.42 μg ($n = 7$) during the same interval. Throughout the next 5-min interval (10–15 min after stimulation), the clusters in hyperosmotic medium were electrically silent, and the attenuation of ELH release was more pronounced. The mean release of ELH in hyperosmotic ASW at this interval was 0.01 μg ($n = 2$) as compared to 0.64 ± 0.21 μg ($n = 7$) in isosmotic ASW. Afterdischarges were also prematurely terminated when ASW was replaced with hyperosmotic ASW solution D (sodium chloride; $n = 2$) or solution C (choline chloride; $n = 2$) (*results not shown*). In experiments in which the ganglion was preincubated in hyperosmotic ASW solutions B (mannitol; $n = 4$) or solution C (choline chloride; $n = 2$) prior to electrical stimulation, no afterdischarges could be evoked (*data not shown*).

Because increasing the osmolality of the medium using a variety of different solutes inhibited the afterdischarge and ELH secretion, it is likely that the inhibition results from the increases in osmolality

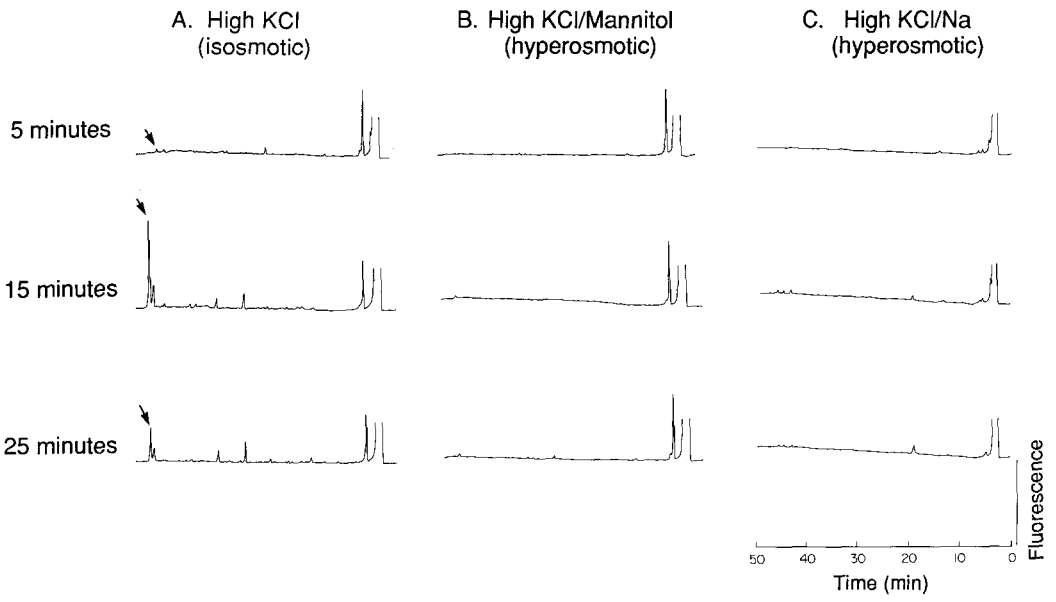

Fig. 3. HPLC profiles from ganglia perfused with (*A*) isosmotic high potassium solution F, (*B*) hyperosmotic high potassium solution H (mannitol) or (*C*) hyperosmotic high potassium solution G (sodium chloride). Arrows designate ELH peaks. *X* axis represents time on HPLC gradient; *Y* axis represents relative fluorescence.

Table 2. Hyperosmotic media inhibit potassium-evoked release of ELH

High potassium media		ELH release (μg)	
F	(isosmotic)	1.61 ± 0.71	(n = 5)
G	(hyperosmotic, sodium chloride)	0.09 ± 0.03	(n = 4)
H	(hyperosmotic, mannitol)	0.05 ± 0.02	(n = 4)
I	(hyperosmotic, choline chloride)	0.05 ± 0.02	(n = 3)

Mean release of ELH for ganglia depolarized by elevation of high potassium in solutions F-I. Release is expressed in micrograms ± SEM.

per se, rather than pharmacological effects specific to the solutes. In these experiments, however, it is not possible to determine whether the ability of the bag cell neurons to secrete peptides in response to depolarization is attenuated, or whether secretion stops because of the inability of the neurons to sustain an afterdischarge of repetitive action potentials.

To determine whether hyperosmotic media inhibit secretion in response to a fixed depolarization, we measured the release of ELH from bag cell neurons in response to high external potassium. Figure 3 illustrates the HPLC profiles obtained in response to depolarization of bag cell clusters by isosmotic high potassium medium (solution F). The mean total release of ELH over 30 min in isosmotic potassium was 1.61 ± 0.71 μg (n = 5). Furthermore, the release of ELH evoked by isosmotic high potassium was found to be calcium dependent. No release of ELH was detected when the extracellular and perfusing media contained isosmotic high potassium medium that was devoid of calcium and that contained the calcium chelator EGTA (solution J) (n = 3; *data not shown*).

In contrast to the results obtained with isosmotic high potassium solution F, depolarization with hyperosmotic high potassium media was ineffective in stimulating release (*see* Table 2). As shown in Figs. 3*B* and *C*, hyperosmotic high potassium solutions H (mannitol) or G (sodium chloride) were ineffective in stimulating ELH secretion. Similar results were obtained with hyperosmotic high potassium solution I (choline chloride).

To assess the possibility that elevated potassium concentrations are unable to adequately depolarize the bag cell neurons in hyperosmotic media, we recorded the membrane potential of isolated bag cell neurons in primary culture penetrated with a single microelectrode. Potassium chloride was added to a dish containing either isosmotic ASW or hyperosmotic ASW solution B (mannitol). Figure 4 demonstrates that elevated potassium chloride (final concentration = 100 mM) caused a depolarization in either media. Both neurons depolarized from resting potentials greater than −80 mV to potentials positive to 0 mV.

To test the possibility that hyperosmotic media do not directly block the secretion of ELH from the bag cell neurons but instead somehow impair the diffusion of peptides from their release sites at axonal varicosities to the medium bathing the ganglia, we stimulated release using the calcium ionophore, ionomycin (5–50 μM). Comparable release of ELH was detected in response to these concentrations of

ionomycin in isosmotic and hypersmotic media (*data not shown*). Whether this release induced by ionomycin represents a physiological mechanism is not known. Video enhanced microscopy of isolated neurons in cell culture has revealed morphological changes including retraction of neurites in response to this agent (*unpublished data*). Nevertheless, these experiments clearly demonstrate that the access of ELH released from the bag cell neurons to the bathing medium is not blocked by the hyperosmotic media and that these media inhibit the calcium-dependent release of ELH that normally occurs in response to depolarization with high potassium.

Attenuation of Action Potentials and Calcium Currents by Hyperosmotic Media

As described above, hyperosmotic media inhibit afterdischarges in bag cell neurons. We therefore examined the effects of hyperosmotic media on the ability of these neurons to generate action potentials. Isolated bag cell neurons were penetrated with a single microelectrode and injected with depolarizing current to evoke action potentials. Action potentials in bag cell neurons under these conditions have been shown to be predominantly calcium-dependent, with only a small percentage of cells retaining voltage-sensitive sodium current at early stages in primary culture (Kaczmarek et al., 1982). Voltage traces in response to depolarizing currents of 50–500 pA were recorded before and after exchange with hyperosmotic ASW solution B (mannitol). Figure 5*A* shows a series of five consecutive action potentials elicited by a series of depolarizing pulses in isosmotic ASW. Some frequency-dependent potentiation of the action potential height and width was observed during the train of pulses and has been shown to represent the inactivation of delayed voltage-dependent potassium currents that contribute to the repolarization of the action potential (Kaczmarek & Strumwasser, 1981). Following exchange with hyperosmotic ASW solution B (mannitol), the height of the recorded action potentials was greatly attenuated (Fig. 5*B*). This effect was reversible on returning the cell to isosmotic ASW solution A (Fig. 5*C*). Similar effects were seen in 9 of 10 cells (mean % decrease in height = 25.5 ± 5.4%). In one cell, no change in height was observed. Varying degrees of attenuation of action potentials were seen following exchange with hyperosmotic solution C (choline chloride) (mean decrease = 6.0 ± 3.4%, n = 4), solution D (sodium chloride) (mean decrease = 10.7 ± 0.27%, n = 3), or solution E (sucrose) (mean decrease = 12.2 ± 0.30%, n = 2; in one cell exposed to sucrose, action potential height actually increased by approximately 10%). These results indicate that hyperosmotic media may alter the functioning of voltage-dependent ion channels in bag cell neurons.

The effect of hyperosmolality on voltage-dependent calcium current in isolated bag cell neurons was investigated using a single microelectrode voltage clamp. To minimize space-clamp artifacts, cells with neuritic growth of less than one soma diameter were selected for these experiments. Calcium current was isolated from other currents by replacing isosmotic ASW solution A with isosmotic solutions K (barium) or M (calcium) which contained cesium chloride and tetra-ethyl ammonium bromide (*see* Table 1). These media largely eliminate the voltage-dependent and calcium-dependent outward potassium currents and allow analysis of the inward barium or calcium currents, respectively. Figure 6*A* shows the current traces obtained following depolarization of a neuron in isosmotic barium medium from a holding potential of −60 to 0 mV (left panel). Exchange with a barium-containing hyperosmotic solution L (mannitol) led to a marked attenuation of the inward current (middle panel). The decrease in inward current was apparent as soon as exchange of medium was complete (30–60 sec) and lasted for as long as the hyperosmotic solution was present (for at least 1 hr). Furthermore, the inhibition of inward current occurred at all potentials tested and was reversible upon return to isosmotic barium solution K (right panel). The mean decrease in inward barium current was 66.4 ± 8.6% (n = 6). Figure 6*B* shows the current traces obtained from a different bag cell neuron in which currents were recorded in an isosmotic calcium solution M. As was found for barium current, exchange with calcium-containing hyperosmotic solution N (mannitol) caused an inhibition of voltage-dependent inward current (middle panel). The mean decrease was 66.1% (n =2). Again, the effect of this hyperosmotic medium occurred at all potentials tested and was reversible on return to isosmotic conditions (right panel). In some but not all experiments, small (0–20%) changes in leak currents were also measured after exposure to hyperosmotic media. These changes, however, were not correlated with changes in the calcium current. It appears, therefore, that hyperosmotic media can lead to the attenuation of voltage-dependent inward current in bag cell neurons regardless of whether the charge carrier is barium or calcium.

These data suggest that inhibition of calcium current contributes to the attenuation of action potentials measured in isolated bag cell neurons and may contribute to the termination of the afterdischarge observed in the intact ganglion. Furthermore, blockade of inward calcium current and the expected decrease in calcium influx provides a mechanism

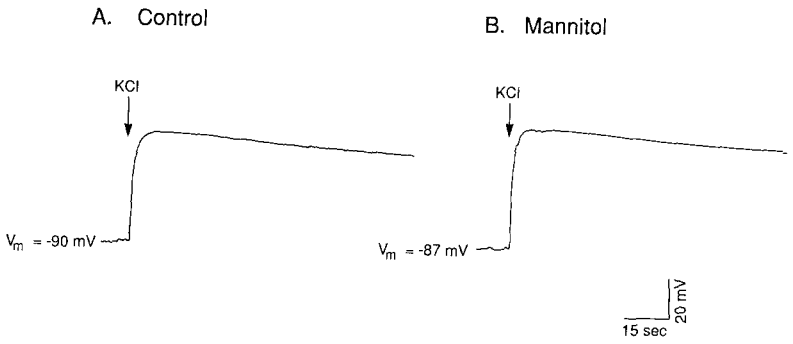

Fig. 4. (*A*) Intracellular recording from an isolated bag cell neuron penetrated with a microelectrode. The bag cell neuron was depolarized from a resting membrane potential of −90 to approximately 0 mV by elevation of external potassium (final concentration of KCl = 100 mM). (*B*) Depolarization of a different bag cell neuron from a resting potential of −87 to greater than 0 mV by elevation of external potassium in hyperosmotic ASW solution B (mannitol).

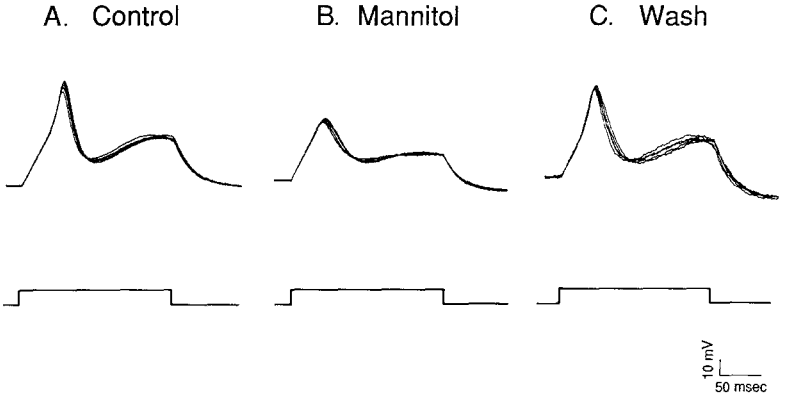

Fig. 5. Intracellular recording from an isolated bag cell neuron. Action potentials were triggered with depolarizing current (500 pA) before and after exchange of isosmotic ASW solution A (*A, Control*) with hyperosmotic ASW solution B (*B, Mannitol*). Attenuation of action potentials by hyperosmotic medium was reversible upon return to isosmotic conditions (*C, Wash*).

by which hyperosmotic media may inhibit calcium-dependent exocytosis of neuroactive peptides in these neurons.

Effects of Hyperosmotic Media on Intracellular Calcium Levels

Further evidence for the inhibition of calcium influx by hyperosmotic media was derived from experiments in which intracellular calcium levels were measured using the calcium-chelating photosensitive dye, fura-2. Following microinjection of fura-2 (free acid) into isolated bag cell neurons in primary culture, cells were depolarized either by current injection or by exposure to elevated external potassium concentrations.

Figure 7 (left top panel) illustrates the basal level of calcium as imaged using fura-2 in a bag cell neuron prior to stimulation. Following a train of depolarizing current pulses (200 pA, 75 msec duration, 4 Hz) a marked increase in intracellular calcium was observed primarily in the neurites (left bottom panel). These results are similar to those described by Fink et al. (1988), and, in the present experiments, were detected in 30 neurites from four cells. The mean resting intracellular calcium level was 297 ± 26 nM, and the mean intracellular calcium level at the end of the train of depolarizing current pulses was 3951 ± 391 nM (n = 30).

The ability of depolarizing current to trigger a rise in intracellular calcium was then examined in the presence of hyperosmotic media (Fig. 7, middle panels). In these experiments, the mean resting level of intracellular calcium in hyperosmotic ASW solution B (mannitol) was found to be 368 ± 27 nM (n = 30) (middle top panel). A train of depolarizing current pulses in the presence of hyperosmotic ASW also caused an elevation in intracellular calcium levels (1723 ± 154 nM, n = 30) (middle bottom panel).

Although a one-way between group ANOVA revealed that calcium levels increase significantly following trains of action potentials ($P < 0.001$), a post-hoc Tukey test clearly demonstrated that the increase in hyperosmotic medium was significantly smaller than in isosmotic medium ($P < 0.01$). No significant difference was found between the basal levels of calcium in the isosmotic and hyperosmotic media.

Calcium entry was also triggered by exposure of the cells to high potassium. As shown in Fig. 8, exchange of isosmotic ASW for isosmotic high potassium solution F caused a significant increase in

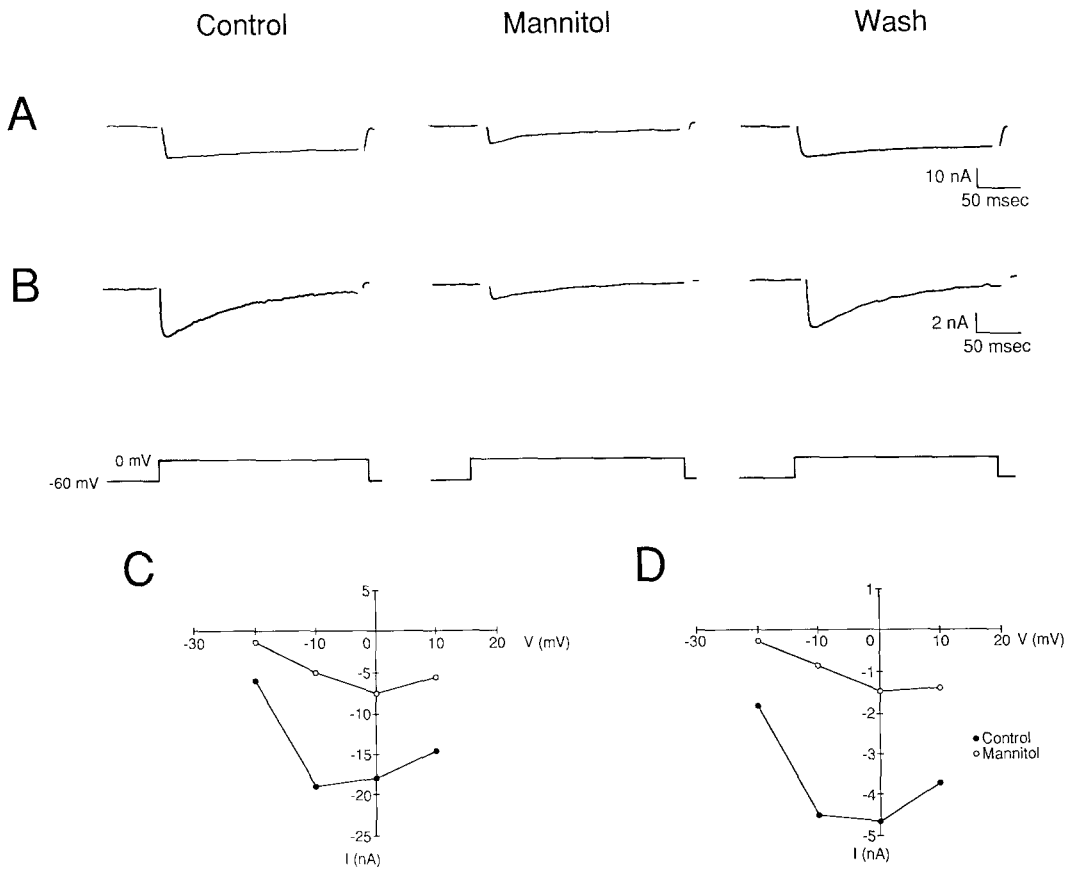

Fig. 6. (*A*) Single microelectrode voltage-clamp traces recorded in an isolated bag cell neuron in isosmotic barium solution K. The neuron was depolarized from a holding potential of −60 to 0 mV for 250 msec and then returned to −60 mV (left panel). Following exchange with hyperosmotic barium solution L, the inward barium current was attenuated (middle panel). The inhibition of barium current by hyperosmotic medium was reversible upon return to isosmotic conditions (right panel). (*B*) A different bag cell neuron in isosmotic calcium solution M was depolarized as in *A* (left panel). Following exchange with hyperosmotic calcium solution N, inward calcium current was attenuated (middle panel). The inhibition of calcium current was reversible on return to isosmotic conditions (right panel). (*c*) *I-V* relation of the peak inward current before (filled circle) and after (open circle) exchange with hyperosmotic barium solution L as described in *A* (*D*) *I-V* relation before (filled circle) and after (open circle) exchange with hyperosmotic calcium solution N as described in *B*.

intracellular calcium levels in the bag cell neurites (left bottom panel). In 19 neurites from four cells, the mean resting intracellular calcium level was 215 ± 30 nM, and within 30 sec after exposure to elevated potassium rose to 2015 ± 185 nM (n = 19). The mean resting intracellular calcium level in hyperosmotic ASW solution B (mannitol) was 238 ± 28 nM (n = 19) (middle top panel). However, depolarization with hyperosmotic high potassium solution H (mannitol) induced an increase to only 757 ± 77 nM (n = 19) (middle bottom panel). As with electrical stimulation, a one-way between group ANOVA showed that depolarization with potassium significantly elevates calcium levels ($P < 0.001$). Post hoc Tukey tests revealed, however, that the increase in hyperosmotic medium is significantly smaller than in the isosmotic medium ($P < 0.01$). Again, hyperosmotic medium was not found to significantly influence basal levels of calcium.

Finally, no apparent rise in intracellular calcium levels was detected in a cell that was exposed to an elevated potassium concentration in 0 Ca^{2+} ASW containing EGTA (solution J, *data not shown*). This is consistent with the total suppression of ELH release from clusters of bag cell neurons under these conditions.

Discussion

Manipulations of extracellular osmolality have been used to define processes that may contribute to excitation-secretion coupling. We now present evidence that in a set of peptidergic neurons, hyperosmotic

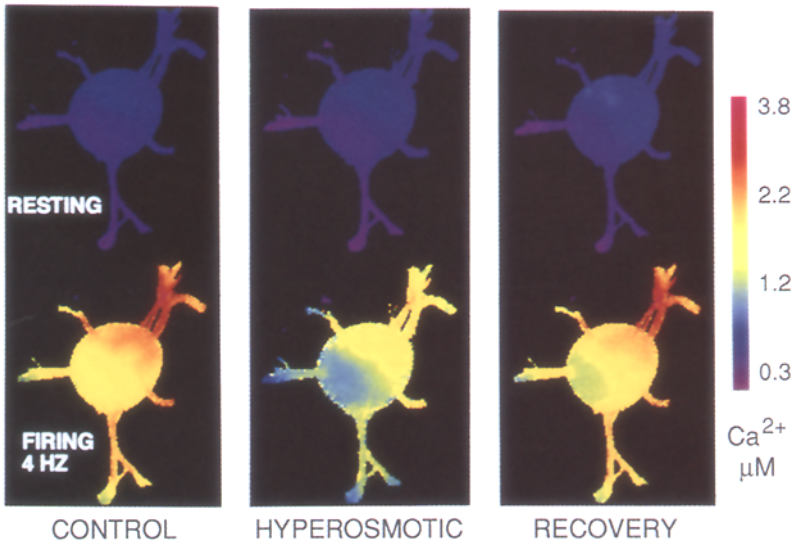

Fig. 7. Effects of electrical stimulation on the intracellular calcium concentration in isolated neurons injected with fura-2. The pictures represent digitized images of the ratio of fluorescence at 340 and 380 nm, converted to approximate calcium concentrations (*see* Materials and Methods). The top left panel shows resting calcium concentration in an isolated bag cell neuron in isosmotic ASW. The bottom left panel shows the elevation in intracellular calcium concentration following a train of ten depolarizing pulses. The middle top panel shows the resting calcium level following exchange with hyperosmotic ASW solution B (mannitol). The middle bottom panel shows attenuation of response to depolarizing pulses in hyperosmotic ASW. The top right panel shows resting calcium distribution upon return to isosmotic ASW. The right bottom panel shows recovery of ability of depolarizing pulses to elevate calcium concentrations under isosmotic conditions.

media attenuates secretion triggered by either action potentials or potassium-evoked depolarization. These media were also found to decrease both calcium current measured under voltage clamp, and calcium accumulation during repetitive depolarizations. The present study demonstrates, therefore, that, as has been found for voltage-dependent potassium channels (Zimmerberg et al., 1990) and anion channels (Zimmerberg & Parsegian, 1986), voltage-dependent calcium channels are sensitive to elevations in osmolality.

In squid giant axon, voltage-dependent delayed rectifier potassium channels undergo a reversible decrease in conductance when exposed to solutions of increased osmolality (Zimmerberg et al., 1990). These solutions did not alter the voltage dependence or ion selectivity of the channels; nor could changes in conductance be attributed to changes in the conductivity of the hyperosmotic solutions. Similar results were obtained in solutions made hyperosmotic with several agents, suggesting that these agents did not act as specific channel blockers. The authors suggested, therefore, that osmotic stress was responsible for the observed effects.

The most likely explanation for our results with the bag cell neurons is that, as with the squid potassium channels, hyperosmotic solutions directly influence the conductance of the voltage-dependent calcium channels. There are, however, some alternative explanations that cannot be ruled out at this time. For example, the changes in calcium current could be secondary to activation of second messenger systems. It has been proposed that cell shrinkage may lead to activation of protein kinase C in lymphocytes (Grinstein et al., 1985); and protein kinase C has been shown to modulate calcium currents in a variety of systems, including bag cell neurons (Strong et al., 1987). However, this mechanism seems unlikely in the present study given that protein kinase C activation is known to enhance, rather than inhibit, calcium current in bag cell neurons (Strong et al., 1987).

A second potential mechanism by which hyperosmotic media could inhibit calcium current is by creating a relative rise in intracellular calcium concentration secondary to a decrease in cell volume. Such a rise in calcium levels could, in turn, lead to calcium-dependent inactivation of calcium current. O'Sullivan and Burgoyne (1988) reported a rise in basal intracellular calcium levels in adrenal medullary chromaffin cells upon exposure to hyperosmotic medium. The mechanism by which intracellular calcium levels were elevated in the chromaffin cells was believed to be secondary to a hyperosmolality-induced increase in intracellular pH which, in turn, elevated calcium levels. In the present study with fura-2, however, we found no statistically significant increases in resting intracellular calcium levels in bag cell neurons upon exposure to hyperosmotic media. In addition, little inactivation of calcium influx occurs during trains of action potentials or voltage-clamp pulses in isosmotic media (Kaczmarek & Strumwasser, 1984), during which time calcium levels rise to about 4 μM (Fig. 7). Thus, it is very unlikely that changes in basal levels of calcium contribute to the attenuation of calcium-dependent influx and secretion.

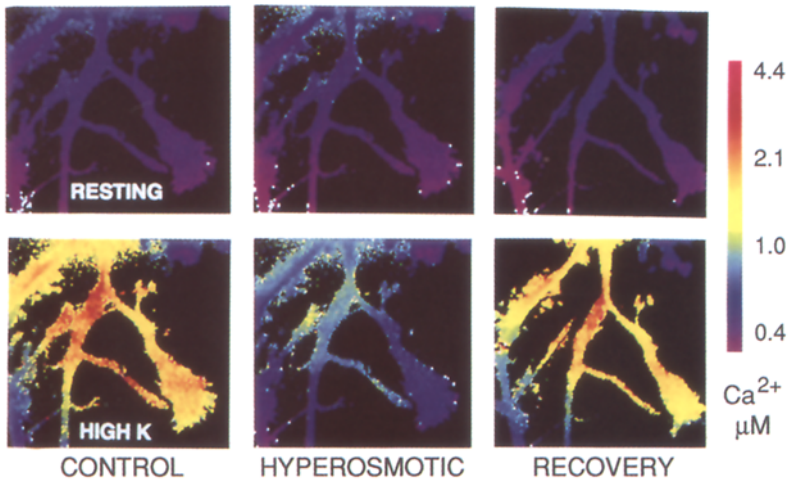

Fig. 8. Effect of elevated potassium on the intracellular calcium concentration of isolated bag cell neurons loaded with fura-2. The top left panel shows resting intracellular calcium distribution as measured using digitized images of the ratio of fluorescence at 340 and 380 nm excitation. Ratios have been converted to approximate calcium concentrations. The left bottom panel shows the increase in intracellular calcium levels following exchange of ASW with isosmotic high potassium solution F. The middle top panel shows resting calcium distribution in hyperosmotic ASW solution B (mannitol). The middle bottom panel shows the attenuation of potassium-induced elevation of calcium concentrations upon exchange with hyperosmotic high potassium solution H (mannitol). The top right panel shows the resting calcium concentration upon return to isosmotic ASW. The right bottom panel shows the recovery of ability of potassium-induced depolarization to trigger rise in intracellular calcium following return to isosmotic conditions.

In summary, the present experiments demonstrate that hyperosmotic media attenuate voltage-dependent calcium currents in bag cell neurons. It is possible that similar effects on vertebrate calcium channels contribute to the neurological changes that can accompany clinical hyperosmotic states such as hyperosmotic hyperglycemic nonketotic coma (Arieff Caroll; 1972, 1974). These data also emphasize the importance of assessing calcium influx when using secretagogues in a hyperosmotic milieu.

This work was supported by NIH Grant NS-18492 to L.K. Kaczmarek.

References

Arch, S. 1972. Biosynthesis of egg-laying hormone (ELH) in the bag cell neurons of *Aplysia californica*. *J. Neurophysiol.* **43**:399–519

Arieff, A.I., Caroll, H.J. 1972. Nonketotic hyperosmolar coma with hyperglycemia: Clinical features, pathophysiology, renal function; acid-base balance, plasma-cerebrospinal fluid equilibrium and effects of therapy in 37 cases. *Medicine* **51**:73–94

Arieff, A.I., Caroll, H.J. 1974. Cerebral edema and depression of sensorium in nonketotic hyperosmolar coma. *Diabetes* **23**:525–531

Berry, R.W. 1988. Alpha bag cell peptide reduces stimulated cAMP levels and pro-ELH synthesis in bag cells. *Mol. Brain Res.* **4**:267–271

Blankenship, J.E., Haskins, J.T. 1979. Electrotonic coupling among neuroendocrine cells in *Aplysia*. *J. Neurophysiol.* **42**:347–355

Brown, E.M., Pazoles, C.J., Creutz, C.E., Aurbach, G.D., Pollard, H.B. 1978. Role of anions in parathyroid hormone release from dispersed bovine parathyroid cells. *Proc. Natl. Acad. Sci. USA* **75**:876–880

Brown, R.O., Mayeri, E. 1989. Positive feedback by autoexcitatory neuropeptides in neuroendocrine bag cells of *Aplysia*. *J. Neurosci.* **9**:1443–1451

Chandler, D.E., Whitaker, M., Zimmerberg, J. 1989. High molecular weight polymers block cortical granule exocytosis in sea urchin eggs at the level of granule matrix disassembly. *J. Cell Biol.* **109**:1269–1278

Chiu, A.Y., Hunkapiller, M.W., Heller, E., Stuart, D.K., Hood, L.E., Strumwasser, F. 1979. Purification and primary structure of neuroactive egg-laying hormone of *Aplysia californica*. *Proc. Natl. Acad. Sci. USA* **76**:6656–6660

Connor, J.A. 1986. Digital imaging of free calcium changes and of spatial gradients in growing processes in single, mammalian central nervous system cells. *Proc. Natl. Acad. Sci. USA* **83**:6179–6183

Fink, L.A., Connor, J.A., Kaczmarek, L.K. 1988. Inositol triphosphate releases intracellularly stored calcium and modulates ion channels in molluscan neurons. *J. Neurosci.* 8:2544–2555

Frazier, W.T., Kandel, E.R., Kupfermann, I., Waziri, R., Coggeshall, R.E. 1967. Morphological and functional properties of identified neurons in the abdominal ganglion of *Aplysia californica*. *J. Neurophysiol.* **30**:1288–1351

Grinstein, S., Cohen, S., Goetz, J.D., Rothstein, A. 1985. Osmotic and phorbol ester-induced activation of Na^+/H^+ ex-

change: Possible role of protein phosphorylation in lymphocyte volume regulation. *J. Cell Biol.* **101:**269–276

Grynkiewicz, G., Poenie, M., Tsien, R.Y. 1985. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.* **260:**3440–3450

Hampton, R.Y., Holz, R.W. 1983. Effects of changes in osmolality on the stability and function of cultured chromaffin cells and the possible role of osmotic forces in exocytosis. *J. Cell Biol.* **96:**1082–1088

Heldman, E., Levine, M., Morita, K., Pollard, H.B. 1991. Osmotic strength differentiates between two types of calcium transport pathways regulating catecholamine secretion from cultured bovine chromaffin cells. *Biochim. Biophys. Acta* **1091:**417–425

Holz, R.W., Senter, R.A. 1986. The effects of osmolality and ionic strength on secretion from adrenal chromaffin cells permeabilized with digitonin. *J. Neurochem.* **46:**1835–1842

Kaczmarek, L.K., Finbow, M., Revel, J.-P., Strumwasser, F. 1979. The morphology and coupling of *Aplysia* bag cells within the abdominal ganglion and in cell culture. *J. Neurobiol.* **10:**535–550

Kaczmarek, L.K., Jennings, K.R., Strumwaser, F. 1982. An early sodium phase and a late calcium phase in the afterdischarge of peptide-secreting neurons of *Aplysia*. *Brain Res.* **238:**105–115

Kaczmarek, L.K., Strumwasser, F. 1981. The expression of long-lasting afterdischarge by isolated *Aplysia* bag cell neurons. *J. Neurosci.* **1:**626–634

Kaczmarek, L.K., Strumwasser, F. 1984. A voltage-clamp analysis of currents underlying cyclic AMP-induced membrane modulation in isolated peptidergic neurons of *Aplysia*. *J. Neurophysiol.* **52:**340–349

Kater, S.B., Mattson, M.P., Cohan, C., Connor, J.A. 1988. Calcium regulation of the neuronal growth cone. *Trends Neurosci.* **11:**315–321

Kauer, J.A., Fisher, T.E., Kaczmarek, L.K. 1987. Alpha bag cell peptide directly modulates the excitability of the neurons that release it. *J. Neurosci.* **7:**3623–3632

Kazilek, C.J., Merkle, C.J., Chandler, D.E. 1988. Hyperosmotic inhibition of calcium signals and exocytosis in rabbit neutrophils. *Am. J. Physiol.* **254:**C704–C718

Knight, D.E., Baker, P.F. 1982. Calcium-dependence of catecholamine release from bovine adrenal medullary cells after exposure to intense electric fields. *J. Membrane Biol.* **68:**107–140

Knox, R.J., Quattrocki, E.A., Connor, J.A., Kaczmarek, L.K. 1992. Recruitment of calcium channels during rapid formation of putative release sites in isolated *Aplysia* neurons. *Neuron* (*in press*)

Ladona, M.G., Bader, M.F., Aunis, D. 1987. Influence of hypertonic solutions on catecholamine release from intact and permeabilized cultured chromaffin cells. *Biochim. Biophys. Acta* **927:**18–25

Loechner, K.J., Azhderian, E.M., Dreyer, R., Kaczmarek, L.K. 1990. Progressive potentiation of peptide release during a neuronal discharge. *J. Neurophysiol.* **63:**738–744

Loechner, K.J., Kaczmarek, L.K. 1989. Hypertonic solutions block peptide secretion and afterdischarges in *Aplysia* bag cell neurons. *Soc. Neurosci. Abstr.* **15:**1275

Loechner, K.J., Kaczmarek, L.K. 1990. Control of potassium currents and cyclic AMP levels by autoactive neuropeptides in *Aplysia* neurons. *Brain Res.* **532:**1–6

Loechner, K.J., Knox, R.J., Connor, J.A., Kaczmarek, L.K. 1991. Modification of calcium current by hyperosmotic media in control and phorbol ester treated neurons. *Soc. Neurosci. Abstr.* **17:**774

Mayeri, E., Rothman, B.S., Brownell, P.H., Branton, W.D., Padgett, L. 1985. Nonsynaptic characteristics of neurotransmission mediated by egg-laying hormone in the abdominal ganglion of *Aplysia*. *J. Neurosci.* **5:**2060–2072

Merkle, C.J., Chandler, D.E. 1989. Hyperosmolality inhibits exocytosis in sea urchin eggs by formation of a granule-free zone and arrest of pore widening. *J. Membrane Biol.* **112:**223–232

Newcomb, R., Scheller, R.H. 1987. Proteolytic processing of *Aplysia* egg-laying hormone and R3-R14 neuropeptide precursors. *J. Neurosci.* **7:**853–863

O'Sullivan, A.J., Burgoyne, R.D. 1988. The role of cytoplasmic pH in the inhibitory action of high osmolarity on secretion from bovine adrenal chromaffin cells. *Biochim. Biophys. Acta* **969:**211–216

Roth, M. 1971. Fluorescence reaction for amino acids. *Anal. Chem.* **43,** 880–882

Rothman, B.S., Mayeri, E., Brown, R.D., Yuan, P., Shively, J. 1983. Primary structure and neuronal effects of alpha bag cell peptide, a second candidate neurotransmitter encoded by a single gene in bag cell neurons of *Aplysia*. *Proc. Natl. Acad. Sci. USA* **80:**5733–5757

Rothman, B.S., Sigvardt, K.A., Mayeri, E. 1985. Co-release of five peptides, ELH, AP, alpha-, beta-, and gamma-BCP, derived from a common precursor protein of the bag cells of *Aplysia*. *Soc. Neurosci. Abstr.* **11:**482

Sato, N., Wang, X., Greer, M. 1991. Medium hyperosmolarity depresses thyrotropin-releasing hormone-induced Ca^{2+} influx and prolactin secretion in GH_4C_1 cells. *Mol. Cell. Endocrinol.* **77:**193–198

Scheller, R.H., Jackson, J.F., McAllister, L.B., Rothman, B.S., Mayeri, E., Axel, R. 1983. A single gene encodes multiple neuropeptides mediating a sterotyped behavior. *Cell* **32:**7–22

Scheller, R.H., Jackson, J.F., McAllister, L.B., Schwartz, J.H., Kandel, E.R., Axel, R. 1982. A family of genes that codes for ELH, a neuropeptide eliciting a stereotyped pattern of behavior in *Aplysia*. *Cell.* **28:**707–719

Sigvardt, K.A., Rothman, B.S., Brown, R.O., Mayeri, E. 1986. The bag cells of *Aplysia* as a multitransmitter system; identification of alpha bag cell peptide as a second transmitter. *J. Neurosci.* **6:**803–813

Strong, J.A., Fox, A.P., Tsien, R.W., Kaczmarek, L.K. 1987. Stimulation of protein kinase C recruits covert calcium channels in *Aplysia* bag cell neurons. *Nature* **325:**714–717

Whitaker, M., Zimmerberg, J. 1987. Inhibition of secretory granule discharge during exocytosis in sea urchin eggs by polymer solutions. *J. Physiol.* **389:**527–539

Zimmerberg, J., Bezanilla, F., Parsegian, V.A. 1990. Solute inaccessible aqueous volume changes during opening of the potassium channel of the squid giant axon. *Biophys. J.* **57:**1049–1064

Zimmerberg, J., Parsegian, V.A. 1986. Polymer inaccessible volume changes during opening and closing of a voltage-dependent ionic channel. *Nature* **323:**36–39

Received 11 October 1991; revised 7 January 1992