Water Regulation by a Presumptive Hormone Contained in Identified Neurosecretory Cell R15 of *Aplysia*

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ABSTRACT   Injection of an homogenate of identified neuron R15 into the hemocele of *Aplysia* produced a weight increase of 3–10% within 90 min. Control injections of several other identified neurons or of seawater, were ineffective. The weight increase occurred even when the animals were maintained in 5% hyperosmotic seawater. The activity of the R15 homogenate was retained after acidification to pH 2 and heating to 100°C; but activity was destroyed by proteolytic digestion with Pronase. Dialysis in cellulose dialysis tubing resulted in a significant loss of activity of the retained material. When the R15 homogenate was subjected to gel filtration on Sephadex G-50 (nominal exclusion limits 1,500–30,000 daltons), activity was present in the partially included volumes, but was absent in the totally excluded or totally included volumes. The data support the notion that R15 contains one or more hormones involved in ionic regulation or water balance. The results of bioassays of R15 extracts subjected to different treatments are consistent with the hypothesis that activity is due to one or more stable polypeptides of relatively low molecular weight.

The identified neuron R15 (Arvanitaki and Chalazonitis, 1958; Frazier et al., 1967) located in the abdominal ganglion of the opisthobranch mollusc *Aplysia* has been extensively studied over the past 20 yr. This neuron is large and easily identified on the basis of its appearance and position in the ganglion. It has been utilized to study both short-term (Barker and Gainer, 1975; Strumwasser, 1971) and long-term (Lickey, 1969; Strumwasser, 1971) rhythmic activity. In addition, synaptic inputs to the cell have been used to investigate synaptic plasticity (Halstead and Jacklet, 1974; Schlapfer et al., 1974). Finally, there are substantial morphological and biochemical data on R15, as well as on a similar neuron in pulmonate molluscs (Gainer, 1972 *a*; Gainer and Barker, 1974; Sakharov and Salánki, 1969; Kerbut and Meech, 1966). A limitation of studies on R15 is that the function of this neuron remains unknown. Jahan-Parwar et al. (1969) and Stinnakre and Tauc (1966, 1969) found that R15 is inhibited by hypo-osmotic solutions presented to the osphradium, an external sensory organ in *Aplysia*, and it has been suggested that R15 may trigger avoidance responses in reaction to hypo-osmotic conditions (Prosser, 1973). Morphological data suggest that R15

may be a neurosecretory cell (Frazier et al., 1967). On the basis of its synaptic input and morphology it has been suggested that cell R15 may be concerned with osmotic regulation (Gainer, 1972 b). In the present study we investigated the possible function on the secretory product of R15 by injecting homogenates of R15 into *Aplysia*. We found that homogenates of the cell body of R15 rapidly produce a weight increase. Homogenates of several other identified cells were ineffective. These data indicate that R15 might be concerned with regulating water content in *Aplysia*. Preliminary characterization of the active substance indicated that activity is associated with one or more stable, relatively low molecular weight polypeptides. A preliminary report of some of these results has been published (Kupfermann and Weiss, 1974).

METHODS

Bioassay

The experiments were performed on *Aplysia californica* (100–250 g) maintained in filtered and cooled (14–15°C) artificial seawater (Instant Ocean). Animals were randomly assigned to different experimental groups. During the experiment, an animal was removed from its home tank, immediately given an injection of either cell homogenate or control, and then weighed. Before weighing, all water was dripped from the animal by holding it out of the water, head down for approximately 30 s. Injections were made into the hemocele in the neck region, utilizing a 26-gauge $^3/8$-inch hypodermic needle. The animal was then placed in a 2-liter Lucite chamber containing 1.5 liters of cool isosmotic, hypoosmotic, or hyperosmotic seawater. The seawater, obtained from the home tank, was made 5% hypotonic by addition of distilled water, or was made 5% hypertonic by addition of NaCl. Every 15 min, over a period of 1.5 h, animals were weighed. A blind procedure was used: the experimenter weighing the animals was unaware of which animals received control and which received experimental injections.

In order to dissect individual neurons (Giller and Schwartz, 1971), the abdominal ganglion was pinned to Sylgard (Dow Corning Corp., Midland, Mich.) and was covered by a drop of cooled 70% ethylene glycol in artificial seawater buffered with Tris-HCl to pH 7.6. The sheath was slit with a sliver of razor, and surrounding cells were teased away with sharp forceps. The cell bodies were transferred to the homogenizer by means of a 25-μl capillary pipette. The neurons used in this study (R15, R14, and R2) could be readily identified on the basis of size, color, and position (Frazier et al., 1967).

Cell homogenates were prepared by grinding two cells at a time in a small ground-glass homogenizer (Micro-metric Instruments, Cleveland, Ohio) in 0.2 ml cool seawater. The volume was brought up to 2 cm³ to provide two 1-ml samples for injection.

Dialysis

Four R15 neurons at a time were homogenized in 0.1 ml seawater. To provide carrier material, the cells were homogenized together with a buccal ganglion. In separate control experiments, it was shown that injections of homogenates of the buccal ganglion alone produce no wieght change. The volume of the homogenate was adjusted to 4 ml with seawater, and the material was centrifuged at 20,000 g for 15 min. Half of the supernatant was stored at 6°C; the other half was dialyzed for 16 h against 4 liters of circulating seawater at 6°C. Dialysis was done in cellulose dialysis tubing with a nominal average pore diameter of 24 Å (D2130, Scientific Glass Apparatus Co., Bloomfield, N.J.). Under our conditions, 4% of bovine serum albumin (mol wt 65,000) was lost from the dialysis tube

after 16 h of dialysis, whereas 36% of soybean trypsin inhibitor (mol wt 22,000) was lost. Animals received 1-ml injections of either nondialyzed control or dialyzed material and were weighed after 90 min in 5% hypo-osmotic seawater.

Gel Filtration

Gel filtration of R15 extracts was carried out at 6°C on Sephadex G-50 (Pharmacia, Uppsala, Sweden; nominal exclusion limits 1,500–30,000 daltons), equilibrated with artificial seawater buffered with Tris-HCl at pH 7.6. Two 1-cm diameter columns were utilized with gel beds of 10 and 55 cm. One-milliliter fractions were collected from the short column, and 2-ml fractions from the long column. Columns were stabilized by running 3 vol of seawater through them before standards and R15 extracts were run. The following marker substances were used for standardization: blue dextran (Pharmacia, Uppsala, Sweden), >50,000 daltons; ovalbumin, 43,000 daltons; soybean trypsin inhibitor, 22,000 daltons; horse heart cytochrome c, 12,000 daltons; pancreatic trypsin inhibitor, 6,000 daltons; glucagon, 3,500 daltons (run separately in 0.02 M NaOH, 0.5 M NaCl); and [^{14}C]leucine, <131 daltons. As has been previously described (Andrews, 1964), there was an approximately linear relationship between the logarithm of the molecular weight of the standards and the elution volume containing the peak concentration of the substance. In order to avoid injecting animals with large quantities of foreign proteins, standards were not run during the filtration of the R15 extracts. To establish whether the column runs were reproducible, standards were run immediately before and immediately after the filtrations. In 85% of the cases, the peak values of the concentration of a given standard were within one tube fraction in different runs. Four to six individual R15 cells together with a buccal ganglion were ground in 0.1 ml of cold (6°C) artificial seawater in a tissue grinder. One-tenth of a milliliter of artificial seawater was added to the homogenate to produce a final volume somewhat greater than 0.2 ml. The material was centrifuged at 20,000 g for 15 min and the supernatant was applied to the column. In the short column, starting at the fraction containing the peak of fully excluded material, each 1-ml fraction was collected and divided into two equal volumes. One of the volumes of each fraction was injected into an animal and the weight change produced was determined after the animal was placed in a 5% hypo-osmotic seawater solution for 1.5 h. The second half of each fraction was stored overnight at 6°C, so that a second bioassay could be done the following day. A similar procedure was followed for the long column, except that adjacent 2-ml fractions were combined in order to reduce the number of animals needed for the assay.

Precise determination of the peaks of activity of a column was hindered by the great variability of our bioassay technique. For the purpose of analysis, we utilized a nonquantitative measure of activity. A given fraction was considered to have produced a positive effect if injection of that fraction resulted in a weight increase greater than 2 SD above the mean weight change produced by previously studied.groups of control animals that received injections of artificial seawater or an homogenate of an identified cell (R14 and R2) which does not produce a weight increase. Control injections (N = 15) produced a mean weight change of −1.9% after 90 mn in 5% hypo-osmotic seawater, with a standard deviation of 2.0. Therefore, a weight increase of 2.0% was considered positive. A similar criterion was also used in other experiments in which a nonquantitative assessment of biological activity was sufficient.

Proteolytic Digestion

Proteolytic digestion was carried out with Pronase (Calbiochem, San Diego, Calif., 45 proteolytic U/mg; 2 mg/ml final concentration) at 37°C for 1 h. The Pronase was

preincubated for 30 min to destroy any contaminating enzymatic activities. Six or eight individual R15 neurons and a pair of buccal ganglia were ground in 0.1 ml of artificial seawater, and 0.2 ml of seawater was added. The material was then divided into control and experimental samples of 0.15 ml; 0.15 ml of 4 mg/ml of Pronase was added to the experimental sample and 0.15 ml seawater was added to the control sample. After incubation, the sample was brought to a volume of 1 ml by addition of seawater. Before bioassay, it was important to eliminate proteolytic activity. This was accomplished by acidifying the samples (control and experimental) with 0.1 ml 1 N HCl/ml. The samples were then allowed to remain at room temperature for 10 min and were centrifuged at low speed. The supernatant was then neutralized with Tris buffer to pH 7.8. Each animal received an injection containing the equivalent of the contents of a single R15 cell body.

RESULTS

Bioassay

Since the spike activity of R15 is inhibited by hypo-osmotic stimuli external to the animal (Jahan-Parwar et al., 1969; Stinnakre and Tauc, 1969), we thought that injection of the secretory product of R15 might interfere with water regulation under hypo-osmotic conditions. Animals were therefore challenged by a 5% hypo-osmotic condition, and the resultant weight changes were measured with and without injection of R15 homogenates. Five animals received R15 homogenates. Three groups of five animals each were run with the following control injections: (a) homogenate of cell R14, a cell that has morphological features suggesting that it is neurosecretory (Frazier et al., 1967), (b) homogenate of cell R2, a neuron that is considered a nonneurosecretory cell, and (c) artificial seawater. All control groups responded approximately the same (Fig. 1 A). Within the first half hour, animals gained a small amount of weight, but despite
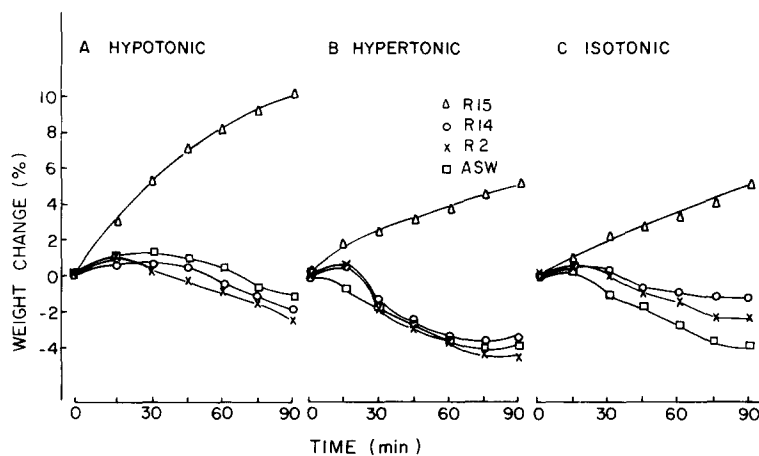

FIGURE 1.   Weight change as a function of time after injection of homogenates of cell R15, R14, R2, and artificial seawater (ASW). Animals were maintained in 5% hypotonic seawater (part A), 5% hypertonic seawater (part B), or isotonic seawater (part C). Each curve represents the mean of five animals.

the hypo-osmotic conditions, they then lost weight and after 1.5 h they weighed 1–5% less than their initial body weight. By contrast, each of the animals receiving an injection of R15 homogenate rapidly gained weight, amounting to 9–12% of initial body weight. At 1.5 h after injection, the difference between experimental and control animals was 14.3%. The weight loss of control animals may reflect a circadian rhythm of weight change, or may be due to repeated handling. We found that uninjected animals in normal seawater also lose 1–5% of body weight after 90 min, when repeatedly weighed every 15 min.

Since the percentage weight gain in animals injected with R15 homogenate was considerably greater than the percentage dilution of the seawater, it is unlikely that the R15 homogenate was simply interfering with volume regulation under hypo-osmotic conditions. Rather, it appeared as if the homogenate was promoting uptake of water into the animal, beyond that which would enter under osmotic forces. To determine if the weight increase produced by R15 homogenate was specific to conditions of hypo-osmotic stress, groups of five animals each were kept under 5% hyperosmotic seawater or in normal seawater. In the hyperosmotic condition, all control animals lost 1–10% weight, but all animals with R15 injections gained 3–8% (Fig. 1 B). At the end of 1.5 h, the mean difference between pooled controls and R15 homogenate animals was 9.2%. Similar results were obtained in normal seawater (Fig. 1 C).

The data for each of the three conditions of water osmolarity were statistically evaluated by measuring the weight change 90 min after the injections. Under each condition, none of the control groups were significantly different from one another ($P > 0.2$ for all three conditions, Kruskall-Wallis one-way analysis of variance). On the other hand, under each condition, the mean weight change of animals injected with R15 homogenate was significantly greater than that of the combined control groups ($P < 0.01$, for all three conditions, Mann-Whitney U test, two tailed).

For the purposes of later experiments, it was desirable to estimate how much the active substance could be diluted and still retain activity. In two experiments, animals were injected with R15 homogenates that were serially diluted. Activity was present (2% weight increase) with 5–10-fold dilution of the contents of a single cell body.

In addition to control injections of identified cells R2 and R14, homogenates of various nerves ($N = 5$ for each nerve tested) of the abdominal ganglion were injected into animals and weight changes were determined. In a few instances, injections of the siphon nerve, branchial nerve, genital nerve, and pleural-abdominal connectives produced weight increase greater than 2%, but the mean weight increase was not significantly different from that produced by artificial seawater. On the other hand, the mean weight increase after injections of homogenates of the pericardial nerve was significantly greater than that after control injections ($P < 0.02$). These results are consistent with the observation that the pericardial nerve always contains an axon of R15 whereas other nerves may also have an axon of R15 but not in all cases (Winlow and Kandel, personal communication). The data are consistent with the notion that the active substance is transported down the axon of R15.

Characterization of Active Principle

The preceding experiments demonstrate that R15 contains a substance that can alter the water content of *Aplysia*. Several laboratories have previously shown that cell R15 in *Aplysia* (Gainer and Barker, 1974; Loh and Peterson, 1974; Wilson, 1974) and the similar neuron, cell 11, in snails (Gainer, 1972a), synthesize one or more low molecular weight polypeptides. We therefore attempted to characterize the active substance of R15 in order to compare it to the putative neurosecretory polypeptides described in this cell.

We first determined whether the activity was localized to the particulate or soluble fraction of the R15 homogenates. In two experiments, R15 homogenate was centrifuged at 105,000 *g* for 0.5 h. The supernatant possessed all the biological activity; the pellet was without activity.

Several experiments indicated that the substance was stable and continued to produce weight changes after a variety of treatments of the homogenate. In two experiments, we found activity present after the homogenate was stored for 12 h at room temperature. Activity was also present after acidification with HCl to pH 2 for 20 min, followed by neutralization to pH 7.8 with Tris-HCl (see Table II), and heating the homogenate to 100°C for 15 min (Table I). The stability of the substance is consistent with the idea that it is a low molecular weight polypeptide.

As a first approach toward estimating the molecular weight range of the substance, R15 homogenates were dialyzed against seawater in cellulose dialysis tubing. The retained material produced a positive effect (greater than 2% weight increase) in three out of four experiments. The average weight gain (2.2%), however, was significantly less ($P < 0.05$) than that produced by the nondialyzed R15 control injections (9.6%). These results suggest that at least part of the activity of the R15 homogenate is associated with a substance with a molecular weight less than 65,000 and probably less than 20,000. The activity that was

TABLE I

HEAT STABILITY

Treatment	N	Weight change (±SE)
		%
R15	3	8.0±1.3
R15, heated	3	7.5±1.0
ASW	3	−1.8±2.0

TABLE II

RESISTANCE TO PROTEOLYTIC DIGESTION

Treatment	N	Weight change (± SE)
		%
R15, acid	5	+5.2±0.8
R15, Pronase, acid	5	−1.8±2.4
ASW	5	+0.5±1.1

Solutions were neutralized to pH 7.8 before injections.

retained either could be due to the same substance, incompletely diffused, or possibly aggregated, or to another active substance with a larger molecular weight.

To obtain a more precise estimate of the molecular weight of the active substance, we utilized gel filtration on Sephadex G-50. In one experiment, we divided the total column effluent into three fractions that were injected into animals. One fraction contained the totally excluded material (>30,000 daltons), one contained the totally included material (<1,500 daltons), and one contained the partially included material (1,500–30,000 daltons). A control animal was injected with artificial seawater. The combined fractions containing the partially included material had strong activity (weight gain 12% greater than control
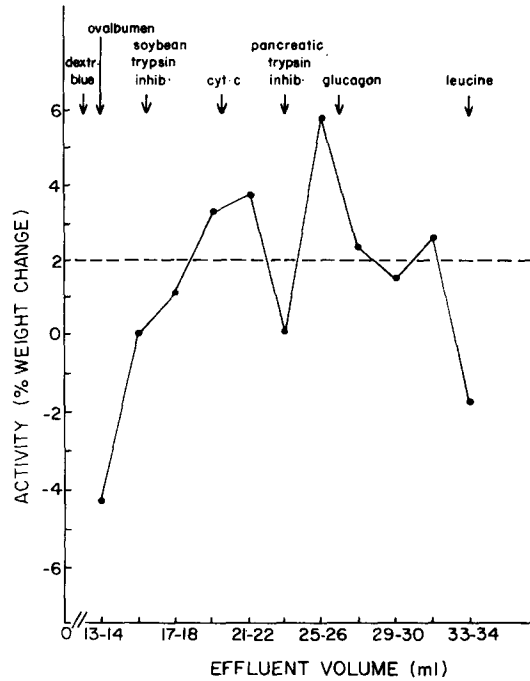

FIGURE 2. Example of weight changes produced by injection of various fractions of homogenate of six R15 neurons filtered through a Sephadex G-50 column. Two-milliliter fractions were collected. Each point represents the weight change produced in an individual animal. Animals initially weighed 86–120 g and received an injection of 0.83 ml/100 g. Dextran blue was added to the R15 homogenate and the first fraction injected (13 and 14 ml) contained the excluded volume, as indicated by the blue marker. The last fraction injected (33 and 34 ml) was the fraction containing the peak of the fully included volume as determined by previous runs with [^{14}C]leucine. Weight changes above the dashed line (2%) are considered positive since they fall 2 SD above the weight changes produced by control injections in previous experiments. The top of the figure indicates different volumes containing the peak concentration of marker substances of various molecular weights run through the column in previous runs with 1-ml fractions.

animal), whereas the other two fractions were inactive (weight gain 1% greater than control). In three additional experiments with the short column, individual 1-ml fractions were assayed. Activity was never present in the totally excluded or totally included volumes. In each experiment, several of the fractions of the partially included volumes produced weight increases greater than 2%, which is 2 SD beyond the mean (−1.9%) of all the control animals utilized in the previous bioassay, under hypotonic conditions. The data obtained with the short column suggest that the active substance has a molecular weight of 1,500–30,000 daltons.

In order to increase the resolution possible with the Sephadex column, three runs were made on a column 50 cm long. Each run provided two sets of determinations of activity. As in the shorter column, activity was not present in the excluded fractions or totally included fractions. Activity (2% or more weight gain) was present in many of the fractions collected from a column (Fig. 2). Individual column runs usually revealed two peaks of activity, but the molecular weight estimate of the two peaks was not consistent and varied from run to run.

Proteolytic Digestion

The previous results suggest that activity of R15 extracts is due to one or more stable substances of a molecular weight of 1,500–25,000 daltons. To determine if the active substance might be a polypeptide, extracts were treated with the proteolytic enzyme, Pronase. The proteolytic activity was then terminated by denaturation with acid, and the extract was injected into animals (see Methods). Control animals received injections of artificial seawater, or acid-treated R15 extracts. Treatment with Pronase completely eliminated the capacity of R15 extracts to produce a weight gain in animals (Table II), whereas control R15 injections were active.

DISCUSSION

The present data support the notion that R15 contains one or more hormones involved in ionic regulation or water balance. The hormone action results in a net increase of water content even in animals that are subjected to an osmotic gradient that would drive water out of the animal (Van Weel, 1957). This suggests that the effect of the hormone is linked in some manner to an active process that regulates water or ion content in the animal.

The finding that the active substance causing net water uptake in *Aplysia* is present in the nerve that contains the main axon of R15 supports the hypothesis that the substance is a factor that is transported from the cell body to distant release sites. Definitive proof, however, that the substance is a true hormone must await the demonstration that it is released into the circulation at concentrations that are physiologically active.

The results of bioassays of R15 extracts subjected to different treatments are consistent with the hypothesis that activity is due to one or more stable polypeptides of relatively low molecular weight. Low molecular weight polypeptides are rapidly synthesized and stored in R15 (Gainer and Barker, 1974; Loh and Peterson, 1974; Wilson, 1974) and in what may be a homologous cell in snails (Gainer, 1972 a). Two peaks of activity have been reported, one associated with a

polypeptide of from 5,000 to 9,000 daltons and a second at 12,000 daltons. Recently, several low molecular species between 1,500–9,000 daltons have been found in cell R15 (Loh and Gainer, 1975). It has been suggested that these polypeptides might be associated with neurosecretory activity (Gainer, 1972 *a*; Loh and Peterson, 1974).

The present results suggest that the active hormone, or hormone and carrier molecule complex has a molecular weight between 1,500 and 25,000 daltons. Our preliminary attempts to utilize gel filtration to specifically characterize the molecular weight of the active substance provided evidence that there may be several species of active molecules. Variability in the position of peak activity in the gel columns precluded definite specification of molecular weight. The variability was not due to a defect in the column, since standards always produced sharp peaks. It is possible that there are several species of active molecules and the variability is due to the inaccuracy of the bioassay. On the other hand, it is possible that there may be only a single species of active molecules and the variability may result from uncontrolled factors related to the material. For example, small variations in experimental conditions may result in different degrees of aggregation or breakdown of the active molecule from column to column. Resolution of these questions would be facilitated by the development of a method of bioassay that produced less variability than the present method.

Since water movement may be a secondary result of ion movement, it is possible that the R15 hormone may be exclusively involved in the regulation of ion movement for functional purposes other than osmotic or water balance. This possibility, however, seems unlikely in view of the demonstration that the firing rate of R15 can be affected by a weak osmotic stimulus applied to the osphradium, a sensory structure exposed to the seawater in which the animal lives (Jahan-Parwar et al., 1969; Stinnakre and Tauc, 1969). Osmotic regulation in *Aplysia* may be particularly important since *Aplysia* is an intertidal animal that can become isolated on the shore or small tide pools for 6 h or longer (Kupfermann and Carew, 1974). Thus, for example, since the animal does not have a protective shell, during exposure to air, it is subject to loss of water. Exposure to air may activate R15, and the hormone released may limit the loss of water, or may promote the uptake of water adhering to the gill or skin. Conversely, since R15 is a spontaneously firing neuron (Frazier et al., 1967; Stinnakre and Tauc, 1969), a decrease of the spontaneous firing rate may promote water loss from the animal, for example in a situation in which the animal is stranded in a tide pool containing hypo-osmotic water. Consistent with this notion is the finding that 4% hypo-osmotic stimuli applied to the osphradium of *Aplysia* inhibit the spontaneous activity of R15 (Jahan-Parwar et al., 1969; Stinnakre and Tauc, 1969).

The present demonstration that R15 contains a substance that affects water content in *Aplysia* should provide a basis for a functional understanding of the physiological properties that have been described for this neuron. Invertebrate neurons are highly advantageous for cellular neurophysiological studies, and several invertebrate neurosecretory systems have already been examined. However, in all previous cases in which the functional effects of a neurosecretory substance are known, the neurosecretory neurons consist of multiple, relatively

small cells. In contrast, R15 is a large cell that can be individually identified. The demonstration of a bioassay for an active substance contained in R15 may facilitate the use of this cell in model studies of the synthesis transport and release of a neurohormone.

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