A Possible Mechanism of Release of Posterior Pituitary Hormones Involving Adenosine Triphosphate and an Adenosine Triphosphatase in the Neurosecretory Granules

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SUMMARY

Neurosecretory granules were isolated from bovine posterior pituitary glands and incubated at 30 or 37° in a medium containing KCl and $MgCl_2$. On exposure to ATP the granules released vasopressin, oxytocin, and protein. Vasopressin release in response to ATP was potentiated by phosphoenolpyruvate + pyruvate kinase, was inhibited by AMP, and was accompanied by a fall in the optical density (at 540 mμ) of the granule suspension. The neurosecretory granules possessed ATPase activity and ATP. It is suggested that ATP and ATPase may participate in the release of hormones from the intact posterior pituitary gland.

INTRODUCTION

As is true of the secretory process in most cells, present knowledge of the cellular events underlying release of the posterior pituitary hormones is fragmentary. It is known, however, that the hormones can be released from the neurosecretory terminals of the posterior lobe of the pituitary gland by excess potassium (1), that this response depends on extracellular calcium (1), is associated with uptake of $^{45}Ca^{++}$ (2), and is blocked by metabolic inhibitors (3) or excess magnesium (1). In these various respects the behavior of the neurosecretory terminals is similar to that of the chromaffin cells of the adrenal gland secreting the medullary hormones (4–7); a further similarity lies in the fact that the two types of hormones are stored within the cell mainly in membrane-limited granules of comparable dimensions—the neurosecretory and chromaffin granules, respectively. In view of this parallelism we considered it of interest to study release of posterior pituitary hormones in the light of recent evidence implicating ATP and ATPase in the release of medullary hormones (8–10). The medullary hormones, to judge from morphological (11, 12) and chemical (13–15) evidence, are released directly from storage granules when the chromaffin cells are stimulated, and it has been known for some time that the membrane of these granules contains an ATPase (16, 17). Although this ATPase has generally been associated with catecholamine uptake (18, 19), it may also participate in catecholamine extrusion (8–10). Thus ATP releases catecholamines and other soluble constituents from isolated chromaffin granules and causes structural changes in the granules, as indicated by light-scattering measurements; both effects are reduced by inhibitors of ATPase.

The present experiments show that ATP has comparable effects on neurosecretory granules isolated from posterior pituitary glands and that these neurosecretory granules contain ATP and an ATPase. A preliminary report of some of the findings has appeared elsewhere (20).

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METHODS

Preparation of Isolated Neurosecretory Granules

Whole pituitary glands were removed from adult cattle of either sex shortly after the animals were killed in the slaughterhouse. The glands were immediately placed on ice and rapidly transported to the laboratory. A sagittal cut was made to divide each gland into right and left halves, and the two halves of the neural lobe were dissected out. Several (usually five) glands were used for each experiment. The fragments of neural lobes were weighed, minced with scissors, and homogenized with ice-cold 0.3 M sucrose (2 ml/gland) with a Teflon pestle and glass tube. The homogenate was then centrifuged at 755 *g* for 10 min, and the supernatant was treated in one of two ways: it was either passed through Millipore filters or subjected to differential and density gradient centrifugation.

Millipore filtration. The 755 *g* supernatant was passed through a Millipore "prefilter" and then through Millipore filters of the following pore size: 3, 1.2, 0.65, 0.45, and 0.3 μ. The final filtrate was centrifuged at 20,000 *g* for 20 min, and the pellet was resuspended in 0.3 M sucrose (1 ml/g original tissue).

Differential and density gradient centrifugation. A purified neurosecretory granule fraction was obtained by the following method [after Dean and Hope (21)]. The 755 *g* supernatant was centrifuged for 10 min at 4340 *g*. The supernatant from this centrifugation was then spun for 10 min at 27,000 *g*. The pellet, which contained the neurosecretory granules, was resuspended in 0.3 M sucrose (1 ml/g original tissue). The granule suspension was then layered on a discontinuous sucrose gradient as described by Dean and Hope: 1.30, 1.35, 1.40, and 2.0 M. After centrifugation at 145,000 *g* for 1 hr, two particulate fractions were discernible, corresponding to fractions A and C of Dean and Hope (21). The former contained the mitochondria, and the latter the neurosecretory granules. These two fractions were removed by a pipette with a curved tip and were used for analysis or incubation.

To prepare lysed granules, the neurosecretory granule fraction from the density gradient was diluted with two parts of distilled water and centrifuged for 20 min at 20,000 *g*. The pellet was resuspended in 2 ml of water and the centrifugation was repeated.

Procedure for Studying Effects of Agents on Release of Granule Constituents

In each experiment 50-μl aliquots of the granule suspension (obtained by the Millipore or the density gradient method) were added to tubes containing 1 ml of a standard incubation medium at 30 or 37°. This medium had the following composition: KCl, 160 mM; NaCl, 5 mM; TES buffer [*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid] (pH 7.0), 10 mM; Mg^{++}, 0.5 mM.

To determine the distribution of granule constituents before experimental treatment, some aliquots were first incubated for 0, 5, or 10 min and then chilled rapidly by swirling the tubes in an ice bath. After preliminary incubation for the same periods, other aliquots were incubated for 0.5–20 min with or without the addition of the substances to be tested for their effect on release of granule constituents. Incubation was then terminated by chilling. After they had been chilled, all tubes were centrifuged at 20,000 *g* for 20 min at 0°. Duplicate or triplicate aliquots were examined in each experiment. The resulting supernatants and pellets were assayed for vasopressin, oxytocin, or protein.

The effect of incubation on the release of posterior pituitary hormones was estimated by comparing the amounts of these substances in supernatants or pellets at the end of the incubation period with the amounts found after preliminary incubation only. A rise in the concentration in the supernatant or a fall in the pellet indicated release. The resulting estimates of release were found to be similar. Results are expressed as percentage of hormone

released per minute. Release of protein was calculated from decrease of the protein content in the pellet.

Analytical Methods

Protein. Pellets obtained by centrifuging neurosecretory granules suspended in incubation medium were first extracted with trichloracetic acid (for estimation of vasopressin) as follows: 1.0 ml of 5% TCA was added, and the mixture was stirred and centrifuged at 20,000 *g* for 5 min. The supernatant was decanted and the extraction was repeated. Protein in the extracted pellet was then solubilized in 0.1 N NaOH and measured by the method of Lowry *et al.* (22). Protein in the neurosecretory granule and mitochondrial fractions of the density gradient was measured directly by the same method.

Posterior pituitary hormones. These were measured by bioassay. Pellets or suspensions were extracted with TCA as described above, and the extracts, neutralized and diluted with saline, were assayed for vasopressin by their pressor effects on the blood pressure of the rat treated with pentolinium (1). Supernatants from incubates were diluted and assayed directly. When the incubation mixtures contained ATP or AMP, a fleeting depressor effect characteristic of these nucleotides (23) was observed before the pressor response. But control experiments showed that this did not interfere with the assay; estimates of vasopressin release made by analyzing supernatants agreed closely with estimates made by analyzing the corresponding pellets, and estimates of total vasopressin (pellet + supernatant) were similar whether granules were incubated alone or with ATP or AMP. The oxytocin content of incubates was measured, after 100-fold dilution with saline, by assay on the rat uterus following the method of Holton (24) with the modifications described by Dean and Hope (21). Control experiments showed that in this dilution the incubation medium, which contained potassium or potassium + ATP, did not interfere with the assay.

Adenosine triphosphatase activity. Activity of ATPase in the neurosecretory granule fraction was assayed by introducing 20 μl of a suspension of granules obtained by the density gradient method (the granules were sometimes intact, sometimes lysed) to 1 ml of a medium (Table 2) containing ATP and measuring the release of inorganic phosphate (P_i) on incubation at 37° for 30 or 60 min. At the end of this period the reaction was terminated by adding 0.2 ml of 30% TCA and chilling in an ice bath. For blank determinations the suspension of granules was added after the TCA. The acidified and chilled reaction mixture was centrifuged at 20,000 *g* for 5 min, and P_i in the supernatant was measured by the method of Martin and Doty (25).

ATP. ATP was measured in both the neurosecretory granule fraction and the mitochondrial fraction of the density gradient. Fractions were treated with equal volumes of 10% TCA and centrifuged at 20,000 *g* for 5 min. The supernatant was extracted three times with equal volumes of diethyl ether and neutralized with $NaHCO_3$. The neutralized extracts were then analyzed for ATP by the firefly method (26).

Fumarase. The method of Racker (27) was used to measure fumarase activity in granule and mitochondrial fractions of the density gradient.

Optical Density Measurements

Fifty microliters of granule suspension (prepared by the Millipore or density gradient method) were added to 1 ml of the standard incubation medium, and the optical density (OD) at 540 mμ was measured in a Zeiss PMQ II spectrophotometer. After about 10–15 min, agents to be tested were added in a volume of 20 μl; OD was measured over the next 30 min or so.

Drugs and Chemicals

Pitressin standard was obtained from Parke Davis & Co., pentolinium from Wyeth, and TES from Calbiochem. ATP, AMP, phosphoenolpyruvate, and pyruvate

kinase were obtained from Boehringer Mannheim, New York.

RESULTS

Stimulant Effect of ATP on Release of Vasopressin and Oxytocin

"Spontaneous" release of vasopressin from neurosecretory granules suspended in the standard incubation medium ranged from 0.5 to 2.0% of the granule-bound hormone per minute in 28 experiments in which incubation was carried out at 30 or 37° for 0.5–20 min. In the presence of ATP (0.5 mM) with or without an ATP-regenerating system (28)—pyruvate kinase (1 μg/ml) + phosphoenolpyruvate (5.0 mM)—the rate of vasopressin release was much higher in each of 23 experiments using granules prepared by Millipore filtration, and in each of 8 experiments using granules prepared by density gradient centrifugation. Especially high values were obtained when release was measured over shorter incubation times at the higher temperature. For example, granules incubated with ATP and the regenerating system at 37° released vasopressin at a

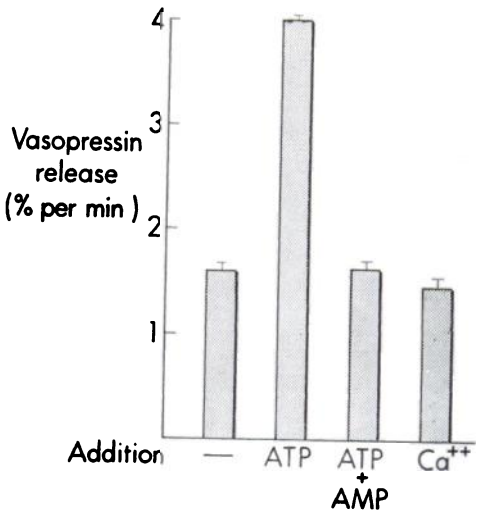

FIG. 2. *Effect of ATP, AMP, and Ca^{++} on release of vasopressin from neurosecretory granules*

Neurosecretory granules were incubated in the standard medium for 5 min at 30°. Additions were made in the following concentrations: ATP, 0.5 mM; AMP, 5.0 mM; Ca^{++}, 1.0 mM. The ordinate shows the percentage of granule-bound vasopressin released per minute. The vertical line above each bar represents the standard error of replicate samples.

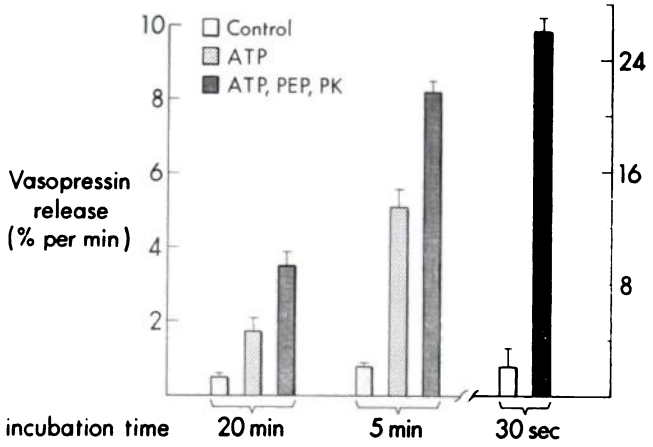

FIG. 1. *Stimulant effect of ATP alone or with phosphoenolpyruvate and pyruvate kinase on release of vasopressin from neurosecretory granules*

Three preparations of neurosecretory granules were incubated in the standard medium at 30° for 20 min or at 37° for 5 min or 30 sec. Additions were made in the following concentrations: ATP, 0.5 mM; phosphoenolpyruvate (*PEP*), 5.0 mM; pyruvate kinase (*PK*), 1 μg/ml. The ordinates represent the percentage of granule-bound vasopressin released to the incubation medium per minute. Note that the values for the 30-sec incubation are plotted on a reduced scale. The vertical line above each bar represents the standard error of replicate samples.

rate in excess of 25%/min. Three representative experiments are depicted in Fig. 1.

Release of oxytocin was measured in a single experiment and a similar result was obtained: "spontaneous" release measured over 10 min at 37° was 0.14 ± 0.10%/min, and release in the presence of ATP plus the regenerating system under the same conditions was 4.54 ± 0.42%/min.

The stimulant effect of ATP (0.5 mM) on vasopressin release was inhibited by the simultaneous addition of AMP (5.0 mM) in each of two experiments, one of which is shown in Fig. 2. This figure also shows that calcium (1 mM) did not increase the rate of vasopressin release from the neurosecretory granules.

Stimulant Effect of ATP on Release of Protein

In addition to stimulating the release of posterior pituitary hormones, ATP also released protein from neurosecretory granules prepared by either method (Table 1).

Effect of ATP on Optical Density of Granule Suspensions

The optical density at 540 mμ of neurosecretory granules suspended in the standard medium at room temperature (about 22°) fell rapidly when ATP (0.5 mM) was added. This effect was potentiated by phosphoenolpyruvate plus pyruvate kinase (Fig. 3).

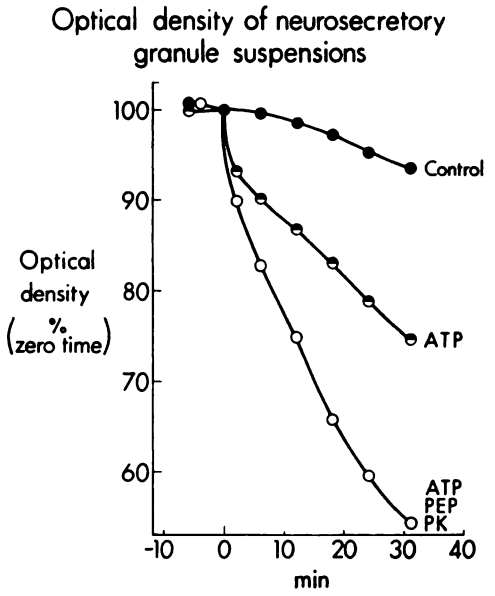

FIG. 3. *The effect of ATP [alone and with phosphoenolpyruvate (PEP) and pyruvate kinase (PK)] on the optical density of neurosecretory granule suspensions*

Aliquots of neurosecretory granules prepared by Millipore filtration were suspended in standard medium; the following additions were made at zero time: ●, none; ◓, ATP (0.5 mM); ○, ATP (0.5 mM), PEP (5.0 mM), PK (1 μg/ml). The ordinate represents the optical density (measured at 540 mμ) expressed as a percentage of the value at zero time

TABLE 1

Effect of ATP + phosphoenolpyruvate (PEP) + pyruvate kinase (PK) on release of vasopressin and protein from neurosecretory granules

Isolated neurosecretory granules were incubated in the standard medium with or without ATP + PEP + PK (0.5 mM, 5.0 mM, 1 μg/ml). In Experiment 1, the granules were isolated by Millipore filtration and incubated for 20 min at 30°. In Experiment 2, the granules were obtained by density gradient centrifugation and incubated for 15 min at 37°. Each result is the mean of two samples.

Expt. No.	Treatment	Release: Vasopressin (mU)	Release: Protein (μg)	Ratio of vasopressin to protein
1	Control	257	19	
	ATP, PEP, PK	1412	67	
	Difference	1155	48	19.9
2	Control	560	41.5	
	ATP, PEP, PK	920	57	
	Difference	360	15.5	23.2

Composition of Neurosecretory Granules

Protein. The mean protein content of eight preparations of neurosecretory granules obtained by the density gradient method was about 100 μg/unit of vasopressin. When expressed in the conventional manner, i.e., units of vasopressin per milligram of protein, a value of 9.94 ± 0.57 was obtained. This may be compared with the corresponding value of 10.73 ± 1.74 units/mg reported by Dean and Hope (21). Three preparations of neurosecretory granules obtained by Millipore filtration yielded a value of 8.54 ± 0.08 units/mg. More than half the protein of the neurosecretory granules was released when the granules (obtained by the density gradient method) were diluted with 10 volumes of distilled water and centrifuged at 20,000 *g* for 20 min. In five experiments 62.7 ± 7.3% of the total protein was recovered in the supernatant and was soluble under these conditions.

ATPase. ATPase activity was found in each of 13 preparations of neurosecretory granules obtained by the density gradient method. In some of these experiments the granules were first lysed, as described in METHODS: all the ATPase activity was found in the insoluble residue. The ATPase activity was supported by magnesium or calcium and inhibited by AMP (Table 2).

ATP. This nucleotide was found in each of seven preparations of neurosecretory granules isolated by density gradient centrifugation. ATP was present in a concentration of about 1 nmole/mg protein. The molar ratio of vasopressin to ATP in

TABLE 2

ATPase activity of neurosecretory granules

Protocol I: 30 min; ATP, 2.0 mM; $MgCl_2$, 2.0 mM; KCl, 30 mM; TES (pH 7.0), 20 mM. Protocol II: 60 min; ATP, 0.5 mM; KCl, 30 mM; TES (pH 7.0), 20 mM. Protocol III: 60 min; ATP, 0.5 mM; $MgCl_2$, 0.5 mM; KCl, 160 mM; NaCl, 5 mM; TES (pH 7.0), 10 mM. Protocol IV: 30 min; ATP, 2.0 mM; KCl, 30 mM; TES (pH 7.0), 20 mM. All incubations were carried out at 37° and were terminated by adding ice-cold TCA to give a final concentration of 5%. Each value is the mean of duplicate determinations.

Experiment	Protocol	Additions to protocol	Granule preparation	ATPase activity (μmoles P_i/mg protein/hr)
1	I	—	Lysed	6.22
2		—	Lysed	4.12
3		—	Lysed	5.68
4		—	Intact	2.12
5		—	Intact	2.86
6		—	Intact	2.88
7		—	Intact	1.54
8		—	Intact	1.82
9		—	Intact	1.59
10a	II	Mg^{++} = 0.5 mM	Lysed	3.97
10b		Ca^{++} = 0.5 mM	Lysed	3.07
10c		Mg^{++} = Ca^{++} = 0.5 mM	Lysed	3.64
11	III	—	Lysed	2.80
12a		—	Intact	1.48
12b		AMP = 5.0 mM	Intact	0.55
13a		—	Intact	1.16
13b		EDTA = 5.0 mM	Intact	0.01
14a	IV	Mg^{++} = 2.0 mM	Intact	2.33
14b		Ca^{++} = 2.0 mM	Intact	0.86
14c		—	Intact	0.48
14d		EDTA = 0.4 mM	Intact	0.05

TABLE 3

ATP content of neurosecretory granules

ATP, vasopressin, and protein were determined in seven different preparations of neurosecretory granules obtained by density gradient centrifugation.

Expt. No.	ATP (nmoles/mg protein)	Vasopressin: ATP (moles/mole)*
1	0.95	19.2
2	0.49	30.2
3	1.34	15.8
4	1.43	16.4
5	1.11	16.2
6	1.14	17.5
7	0.89	17.1
Mean ± SE	1.05 ± 0.12	18.9 ± 1.9

* Assuming 500 units of pressor activity per milligram in pure arginine-vasopressin (53).

the granule fraction was about 20:1. The individual values are given in Table 3.

Both ATP and ATPase are known to be present in mitochondria, and bovine neurosecretory granules prepared by the density gradient method contain traces of mitochondria (21). However, neither the ATP nor the ATPase in the neurosecretory granule fraction can be accounted for by mitochondrial contamination. In the neurosecretory granule fraction the ratio of ATP to fumarase (a mitochondrial marker) ranged from 3.5 to 89 times greater than the mitochondrial fraction, and the ratio of ATPase to fumarase in the neurosecretory granule fraction ranged from 1.4 to 130 times greater than in the mitochondrial fraction. The higher ratios of ATP to fumarase and of ATPase to fumarase in the neurosecretory granule fraction are all the more significant when it is borne in mind that the mitochondrial fraction of the density gradient of Dean and Hope contains a significant amount of neurosecretory granules (21, 29). Thus, in *pure* mitochondria the ratios of ATP and ATPase to fumarase must be lower than in the mitochondrial fraction.

DISCUSSION

The effect of ATP in releasing hormones and protein from the neurosecretory granules of the posterior pituitary gland, which is accompanied by a fall in the optical density of the granule suspension, resembles the effect of ATP on chromaffin granules from the adrenal medulla. It has been proposed that ATP exerts its effect on chromaffin granules by interacting with an ATPase present in the granule membrane to cause some conformational change that allows hormones (8–10) and protein (8, 9) to escape. A similar explanation may account for the action of ATP on neurosecretory granules; our results show that neurosecretory granules possess ATPase activity, which, like that of chromaffin granules, is insoluble in water and presumably resides in the membrane; when this ATPase is inhibited by AMP, the releasing effect of ATP is also inhibited. The protein released along with the posterior pituitary hormones is probably the carrier protein neurophysin, which is the principal soluble protein of the neurosecretory granules (29). In the neurosecretory granules there are close to 20 units of vasopressin per milligram of neurophysin (29), and in our experiments ATP released vasopressin and protein in a similar ratio. Here again there is a parallel with the effects of ATP on chromaffin granules, where catecholamines and soluble proteins are released in the proportion found in untreated granules (8).

A further parallel lies in the present evidence that the neurosecretory granules contain ATP just as do the chromaffin granules. One view is that ATP within the chromaffin granules participates, along with protein, in the formation of a hormone storage complex (30), and it is possible that the ATP in neurosecretory granules functions similarly. However, the amount of ATP relative to hormone is much lower than in the chromaffin granules: the molar ratio of ATP to vasopressin is about 1:20 compared with a ratio of ATP to catecholamines of about 1:4. Calculations presented elsewhere (20) indicate that the negative charges that could be provided by the ATP within the neurosecretory granules are far fewer in number than would be required to neutralize the positive charges on the

posterior pituitary hormones, whereas in the chromaffin granules the opposite charges on ATP and catecholamines are approximately in balance. This raises the question whether the ATP in the neurosecretory granules has some other function. The discovery of ATP in these granules shows that this substance is not unique to granules containing amines, as might have been suspected from reports that ATP is present in chromaffin granules (31), synaptic vesicles from adrenergic nerves (32), and the granules storing 5-hydroxytryptamine in platelets (33). Perhaps ATP is a constituent common to secretory granules storing polypeptides and proteins as well as amines and has some function other than to bind secretory products. As suggested below, it may participate in the release of these products.

This leads us to consider the relevance of the information obtained from the isolated neurosecretory granules to the physiological mechanism for discharge of hormones from the intact posterior pituitary gland. There are now strong grounds for believing that the neurosecretory fibers that make up the hypothalamo-hypophyseal tract have the electrophysiological properties of conventional neurons and that action potentials discharged down this tract in response to osmotic or other stimuli from the perikarya in the hypothalamus serve as the immediate stimulus for release of posterior pituitary hormones from the terminals in the neural lobe: neurosecretory cells generate impulses (34, 35), and isolated neural lobes release posterior pituitary hormones in response to electrical stimulation (1). The events linking arrival of the action potential with release of hormones seemingly include depolarization and calcium entry: the effect of electrical stimulation can be mimicked by raising the extracellular potassium concentration (1), release of hormones is critically dependent on extracellular calcium (1), and increased calcium uptake accompanies release (2). According to some authors ATP is released from electrically excitable membranes when they are depolarized (36, 37). If this is so, and some of the ATP is released inward, then an effect of ATP of the sort observed on granules *in vitro* could occur on the granules *in situ*. Conceivably the ATP within the granules could be released along with the hormones and protein, as occurs in chromaffin granules (8, 13), and this in turn could reinforce release by a positive feedback mechanism. The function of calcium entry in the terminal might be to facilitate approximation of the neurosecretory granules to the plasmalemma by neutralizing mutually repulsive negative charges on the surfaces of the two membranes, as has been suggested for the chromaffin cell (38). This approximation would allow granule ATPase to interact with membrane ATP. In unpublished experiments we have found by electrophoretic means that the neurosecretory granules carry a net negative surface charge that is neutralized by addition of calcium. Such a function for calcium would be consistent with the present evidence, and with that of others (39), that calcium by itself does not cause release of posterior pituitary hormones from isolated neurosecretory granules.

Our finding that protein is released along with posterior pituitary hormones from isolated neurosecretory granules exposed to ATP is consistent with morphological (40) and chemical (41) evidence from intact posterior pituitary glands suggesting that neurophysin is released along with the posterior pituitary hormones. The hypothesis that the physiological mechanism of release of posterior pituitary hormones involves a direct action of endogenous ATP is in harmony with the earlier finding that inhibitors of ATP synthesis block secretion from the posterior pituitary gland (3).

There are reasons for supposing that interaction of ATP and ATPase in release of granule-bound substances may not be restricted to the adrenal medulla and the posterior pituitary gland. Thus exogenous ATP potentiates release of enzymes from leukocytes (42) and release of histamine from mast cells (43). Substances blocking ATP synthesis also block release in mast

cells (44, 45) and salivary glands (46, 47). Moreover, ATPase activity has been found in secretory granules from a wide variety of cells, including leukocytes (42), mast cells (48), β cells of the endocrine pancreas (49), acinar cells of the parotid gland (50), and nerve endings from brain (51, 52). The presence of ATPase in granules containing neurohumors, the synaptic vesicles, is particularly interesting since the factors known to be involved in the release of neurohumors from nerves so closely resemble those involved in the release of posterior pituitary hormones from neurosecretory fibers. This similarity has already prompted the suggestion that there may be a common mechanism for release of these two types of secretory product (1).

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