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Studies of the Mechanism of Antidiuretic Hormone Release

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Introduction

In recent years, a considerable amount of information has been collected concerning the role of calcium in the release of neurohypophysial hormones. Also, the possible role of ATP has been studied to some extent. The hypothalamo-neurohypophysial system in these respects can probably be taken as a model system for neurosecretion.

The authors here take the liberty of mainly reporting new results of their own. Several reviews of previous experiments on these problems have been published during the past few years (1, 8, 9, 18–21).

Movement of Calcium into the Neurosecretory Nerve Endings on Stimulation and Reestablishment of a Normal Low Intracellular Concentration of Free Calcium

It seems now well established that on stimulation of the neurosecretory cells a trigger amount of calcium moves over the cell membrane, probably through special calcium 'channels' (7, 10–12, 16, 17) and induces release of hormone, probably by exocytosis. The intracellular concentration of free calcium is presumably very low (of the order of 10^{-7} *M*) (18). For this system to be able to work, there must exist mechanisms which normalize the increased intracellular concentration of free calcium after stimulation. These processes involve transport of calcium out over the cell membrane, probably at least some of it being dependent on sodium transport the opposite way. The problem of the existence of a calcium 'pump' of the type known, e.g. in erythrocyte cell membrane, has not yet been solved. In addition, various sequestrating mechanisms are able temporarily to reduce the axoplasmic concentration of free calcium. This especially involves mitochondria, which have an ATP-dependent calcium uptake (13).

We have previously (21) found that isolated secretory granules contain a high concentration of calcium. The granules were prepared at low temperature and the calcium concentration may be higher if the preparation is made at more normal temperatures. We have demonstrated an ATP-dependent uptake of calcium by such granules. It was small when expressed per unit granule protein (13). If one, however, expresses it per unit granule membrane protein, it naturally becomes much higher. The granule preparation only took up calcium for a short time, then the granules burst. This is probably due to an ATPase-dependent proton (and chloride) transport into the granules with ensuing osmotic effect. Such a mechanism has been suggested for catecholamine-containing granules (2).

Sequestration might also involve binding to endoplasmic reticulum, e.g. a binding to proteins of the same character as those which have been demonstrated to be present in the sarcoplasmic reticulum.

Recently, a number of analogies have been pointed out between stimulus-secretion coupling and stimulus-contraction coupling. Muscle biochemistry is much more advanced in these respects than secretion biochemistry (1, 5). It would seem appropriate to apply some of the findings and ideas of muscle biochemistry to secretion biochemistry since so many functional analogies can be drawn. This has recently been done concerning calcium-binding proteins. Striated muscle contains proteins with a high affinity for calcium (5) which seem to have a clear function in contraction. In addition, there are also present in muscle tissue proteins with a lesser affinity, such as modulators of cyclic nucleotide phosphodiesterase and inhibitors of such modulators (6, 23).

We have recently started *a series of studies on isolation of calcium-binding proteins from ox neurohypophyses.* So far, we have isolated one in a high degree of purity from the soluble fraction (14). Its molecular weight was approximately 15,000. The purification procedure involved ammonium sulphate fractionation, DEAE-cellulose chromatography and gel filtration on Sephadex G-100 and Sephadex G-50. Conventional and SDS polyacrylamide gel electrophoresis demonstrated it to be a protein distinct from the S-100 protein and the soluble hormone-binding proteins (neurophysins) abundant in the neurohypophysis. It appears to be the only calcium-binding protein in the soluble part of the homogenate. It has an apparent K_{diss} for calcium of 1.1×10^{-5} *M* (at 22 °C) and a binding capacity of 2 mol of calcium per mole of protein. This protein may be identical to the calcium-dependent modulator of cyclic nucleotide phosphodiesterase, isolated by *Lin et al.* (6) from bovine brain. It may also be identical to similar proteins isolated from adrenal medulla and the electroplax of the electric eel. From the microsomal fraction from ox neurohypophyses, we have (after solubilization) isolated a calcium-binding protein which in some respects had somewhat similar properties (binding capacity and dissociation constant) as the one isolated from the soluble fraction. These proteins may be identical. Mem-

brane-bound calcium-stimulated ATPases have been isolated from ox neurohypophyses (22). It would appear unlikely that the 2 proteins with molecular weights of approximately 15,000 are fragments of such ATPases. However, from the deoxycholate fraction also a protein with a molecular weight of approximately 68,000 was isolated. Calcium-binding proteins were also present in the deoxycholate *in*soluble fraction. The latter group of calcium-binding proteins all need further characterization. There may be proteins with a really high affinity for calcium among them. These proteins might serve crucial functions in the release process, e.g. stimulated by the trigger amounts of calcium entering the cells.

Mechanism of Action of Trigger Calcium

Recently, Drs. *Dahl and Gratzl* from Homburg, West Germany, and Dr. *Russell* and I, in our laboratory, have studied problems of cation specificity and concentration dependence of calcium-induced fusion of isolated neurosecretory vesicles from ox neurohypophyses. The essential features of the studies (27) were that isolated secretory vesicles were incubated in a very simple medium (Na-cacodylate 10 m*M*, pH 7.0; sucrose 250 m*M*, and EGTA 1 m*M*) and that the effects of increasing the calcium concentration in the medium from less than 10^{-7} to 10^{-3} *M* on fusion of granules were studied. Fusion was studied by freeze fracture electron microscopy which especially demonstrates aspects of the membranes in the preparations. With increasing calcium concentration, an increase of fusion was observed with a half-maximal effect between 10^{-6} and 10^{-5} *M* calcium. The changes could not be produced by magnesium or strontium. These morphological changes of neurosecretory vesicles were thus similar to changes demonstrated by *Dahl and Gratzl* (3, 25, 26) in other secretory tissues, such as pancreatic islets, liver and adrenal medulla. In the present experiments in addition, it was shown that in parallel to the increased fusion there was an increased release of vasopressin to the medium, with a similar concentration dependence and ion specificity. The vesicle preparations contained sheets of plasma membrane and the increased hormone release observed might occur by exocytosis-like processes.

Studies of ATP-Induced Emptying of Neurosecretory Vesicle Content of Vasopressin

Another important aspect of vasopressin release is the fact that it has been reported to be dependent on energy. This was already found by *Poisner and Douglas* (4) in 1968 in experiments where isolated neurosecretory granules released vasopressin, oxytocin, and protein on exposure to ATP. They also ob-

served ATP-splitting activity associated with a purified granule preparation. They suggested that ATP and ATPase may participate in the processes leading to the release of hormones from the neurohypophysis. *Warberg and Thorn* (24) repeated the experiments of *Douglas and Poisner* and found essentially the same results. However, they stressed the fact that the apparent dependence of release on energy might involve other processes than the release step itself, i.e. that energy was necessary to keep the activation machinery intact.

Vilhardt and Hope (22), on the other hand, have been unable to find ATPase activity associated with the neurosecretory granules. *Russell and Thorn* (15) have recently investigated the effect of ATP on isolated secretory granules with a view to try to clarify whether the ATP-induced release of granule contents is due to a membrane instability caused by ATP or an enzyme-mediated effect.

It was demonstrated that isolated neurohypophysial secretory vesicles released their content of vasopressin in the presence of ATP. The release was nearly complete. It occurred with a half-maximal ATP concentration of 0.25 m*M*. Calcium was not necessary for the effect. This fact alone shows that this specific type of release is of a different kind than normal release which requires calcium. ADP, AMP, and ITP were shown to mimic the effect of ATP. This fact suggests that ATPase is not involved in this release and that it is rather due to an instability of the vesicle membrane caused by the nucleotides. On the other hand, some involvement of ATPase is possible. The background for this suggestion was the following: The ATP effect was dependent on magnesium. AMP-PCP, a non-metabolizable ATP analog, inhibited the ATP-induced granule emptying. Although this effect might be explained via binding of magnesium by AMP-PCP, this was not sufficient to explain the inhibition. These findings therefore might go along with the hypothesis that there is some ATP splitting in these experiments. This suggestion was actually supported by experiments on phosphorylation of the granule membranes. Utilizing the property of ATP to selectively render the secretory granules lighter, a method was developed to obtain granule 'ghosts' in a highly purified form. These purified membrane ghosts were shown to be phosphorylated in the presence of ATP. After the conclusion of these experiments, an article on ATP-evoked catecholamine release from catecholaminergic granules was published by *Casey et al.* (2). They suggested that the release is driven by an inwardly directed proton-translocating adenosine triphosphatase. A resulting proton-anion influx causes osmotic lysis of the chromaffin granules. They considered it unlikely that there was any involvement of this process in the normal release process. As previously stated, such a mechanism might well be responsible for part of the release found in our experiments on isolated neurohypophysial secretory granules. It would seem that it must be concluded that so far no clear evidence has been presented that ATP is critically involved in the release process itself.

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