70-21,764

RAYNOR, John Lawrence, 1942-
THE ROLE OF CALCIUM IN THE RELEASE OF THE ANTIDIURETIC HORMONE IN ANESTHETIZED DOGS.

The University of Michigan, Ph.D., 1970
Physiology

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THE ROLE OF CALCIUM IN THE RELEASE OF THE ANTIDIURETIC HORMONE IN ANESTHETIZED DOGS

by

John Lawrence Raynor

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy in
The University of Michigan
1970

Doctoral Committee:

Professor Richard L. Malvin, Chairman
Associate Professor Peter H. Abbrecht
Associate Professor James A. Greene, Jr.
Assistant Professor Dorothy S. Luciano
Professor Walter S. Wilde

ABSTRACT

THE ROLE OF CALCIUM IN THE RELEASE OF THE ANTIDIURETIC HORMONE IN ANESTHETIZED DOGS

by

John Lawrence Raynor

Chairman: Richard L. Malvin

Experiments were conducted on the role of calcium in the release of the antidiuretic hormone. In vitro evidence from other laboratories has implicated calcium in the mechanism by which the antidiuretic hormone is released from the neurosecretory axons of the posterior pituitary gland. It is felt that calcium enters these axons during depolarization and inhibits the binding of ADH to protein. The increase in the concentration of free ADH in the cytoplasm would lead to an increased rate of movement of ADH out of the cell. In this thesis an attempt was made to demonstrate that calcium is involved in the release of ADH in intact animals.

All experiments were performed on anesthetized dogs. After control and experimental periods fifty milliliters of blood were taken from the femoral artery for analysis of ADH and plasma sodium, calcium, and osmolality. The ADH content of the plasma was analyzed by a method involving extraction, concentration, and a bioassay. The bioassay involved the measurement of conductivity changes in the urine of hydrated, anesthetized rats. All unknowns were compared to the response to standard doses of vasopressin.

The infusion of calcium chloride into the carotid artery of four dogs caused an increase in the level of plasma ADH. The response was significantly greater than when the same dose was infused in the femoral vein of four other dogs. This indicated that the site sensitive to calcium changes is located in the head.

In an attempt to localize the site in the head where calcium was acting, ventriculo-cisternal brain perfusions were performed in three dogs. Increasing the calcium concentration in an artificial cerebrospinal fluid perfusing the brain did not elicit an increase in plasma ADH. An increase in the sodium chloride concentration of the solution perfusing the brain elicited a significant rise in plasma ADH. These results are consistent with the possibility that the site in the brain sensitive to calcium is farther from the ventricular system than the site sensitive to sodium chloride. Since the pituitary gland is located farther from the ventricular system than the hypothalamus my findings are compatible with the hypothesis that sodium chloride acts on some area of the hypothalamus whereas calcium acts directly on the axons of the posterior pituitary gland.

Thyrocalcitonin was injected in four dogs in order to lower plasma calcium. The decrease in plasma calcium was concomitant with an increase in ADH. This increase in ADH could be explained by the lowering of mean arterial pressure following thyrocalcitonin injection.

The in vitro studies mentioned above indicated that high doses of magnesium inhibited the response to calcium. At moderate doses of magnesium I was not able to inhibit the release of ADH in four dogs.

It can be concluded that calcium is a stimulus for the release of ADH and that the site sensitive to increases in calcium is located in the head. The finding that calcium did not increase ADH when perfused through the ventricular system is compatible with the in vitro evidence that calcium acts directly on the posterior pituitary gland.

ACKNOWLEDGMENTS

I would like to thank Dr. Richard L. Malvin for his kindness and guidance throughout the course of these experiments. I would also like to thank the other members of my committee -- Dr. Peter H. Abbrecht, Dr. James A. Greene, Dr. Dorothy S. Luciano and Dr. Walter S. Wilde, for their time and helpful advice.

The problem of calcium and ADH was originally pointed out to me by Dr. Jean-Philippe Bonjour. Dr. Bonjour, Miss Nina Fitch and Mrs. Linda Tillema were all exceedingly helpful in teaching me the methodology of ADH analysis.

During many of these experiments I was fortunate to have had the excellent technical assistance of Mrs. Kimberly Anderson and Mrs. Tos Vandersteen. I would finally like to thank Mrs. Fran Gervasi, Mrs. Helen Mysyk and my wife Kathy for aid in typing this manuscript.

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INTRODUCTION

Structure of ADH

Although it has been known for some time that pituitary extracts could be used to correct water deficiencies in diabetus insipidus (Magnus and Schaffer, 1901), it was not until 1935 that Stehle and Fraser achieved by a fractional precipitation a highly active preparation. By the early 1950's vasopressin was isolated in a pure enough form for structural studies. The structure and synthesis of arginine vasopressin and lysine vasopressin were accomplished in the laboratory of DuVigneaud (DuVigneaud et al., 1953, DuVigneaud et al., 1954). They were found to be polypeptide chains of nine amino acids which differed in the amino acid in the eighth position. Arginine vasopressin is found in almost all mammalian species including man and dogs. Lysine vasopressin has so far only been found in pigs and hippopotami. The structure of arginine vasopressin is illustrated in Figure 1.

Action of ADH on Kidney

Normally ADH acts on the kidney to decrease the flow of urine and increase urine osmolality. However, the kidney is able to excrete a hypertonic urine in the absence of ADH. This was seen when rats with hereditary diabetes insipidus were dehydrated (Valtin, 1966). Also, dehydrated hypophysectomized animals excrete a urine only slightly hypotonic. On the other hand, McDonald and DeWardner (1965), showed that infusion of saline and vasopressin could result in a hypo-osmotic urine. Thus the kidney has mechanisms in addition to ADH for the regulation of urine flow and concentration.

In unanesthetized rats ADH was found to have no effect on renal blood flow or glomerular filtration rate (Malvin and Fusco, 1967). However, the possibility remains open that ADH causes a redistribution of blood flow in the kidney. A change in medullary blood flow could conceivably change the cortico-medullary concentration gradient and

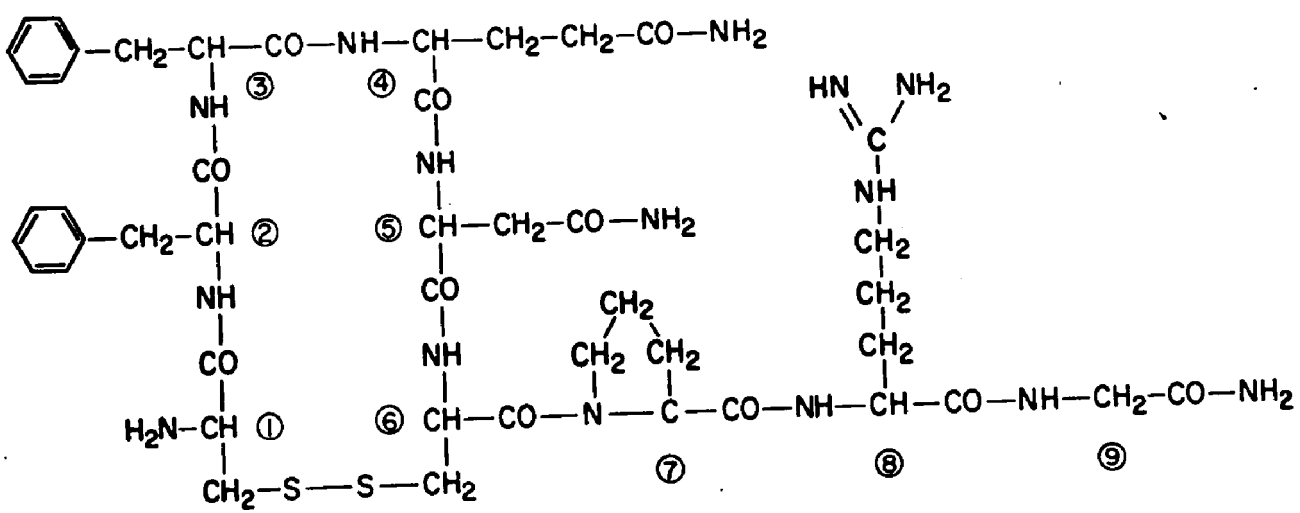


Figure 1 -- The structure of arginine vasopressin.

thus be the cause of a change in urine osmolality. Although the difficulties in measuring blood flow to different areas of the kidney are enormous, there is some indication that ADH might reduce meduliary blood flow (see Thorn, 1968, for review).

ADH does not appear to act on any segment of the kidney tubule except the collecting duct and probably the distal tubule in some animals such as rats (Grantham and Burg, 1966). In addition ADH promotes salt and water reabsorption in the frog skin and toad bladder (Hays and Leaf, 1962). In the absence of ADH almost all of the water delivered to the collecting duct is excreted. In the presence of large amounts of ADH almost all of this water is reabsorbed (see Thorn, 1968, for review).

The manner by which ADH increases the permeability to water is not understood at the present time. Thorn (1968) has proposed the theory that the antidiuretic hormone acts on the kidney by displacing calcium from cell membranes. His theory is based on the fact that calcium inhibited the action of vasopressin. In addition, Thorn (1960) showed that vasopressin produced an increase in calcium excretion which correlated with the length and duration of the antidiuretic response. He proposed that the additional calcium that is excreted is released from cell membranes by ADH. The theory was also tested (Thorn and Schwartz, 1965) by loading toad bladder tissue with Ca^{45} and observing the washout in the presence and absence of vasopressin. The addition of the hormone caused an increase in washout. This increase in washout was influenced by calcium and pH in the medium in the same manner as changes in water permeability. Further support for Thorn's theory is the fact that calcium affects the permeability of the distal part of the nephron to water but not the proximal part. Lassiter (1965) showed by microperfusion that the presence of calcium in the perfusing solution had no effect on the water permeability of proximal tubules but considerably decreased the permeability of distal tubules.

Leaf (1965), using energy considerations and measurements of the permeability of the toad bladder, proposed the theory that vasopressin acts by enlarging the radius of pores and thus increasing the bulk flow of water. Another possibility for the mode of action of vasopressin involves adenyl cyclase as an intermediate (Orloff and Handler, 1964). In toad bladder and dog kidney arginine vasopressin stimulates the production of 3, 5 cyclic AMP and of phosphorylase activity. In addition when 3, 5 cyclic AMP is added to the blood side of the membrane the permeability to water was increased. The concentrations used in these studies were quite high and it is possible that these effects would not be seen at physiological concentrations. Both the calcium theory of Thorn and the adenyl cyclase theory of Orloff and Handler are compatible with the change in pore diameter proposed by Leaf. One only need to propose that the replacement of calcium by vasopressin enlarges pores in the membrane or that increased phosphorylase activity leads to increased pore diameter.

Synthesis and Release from Cell

Sachs (1960, 1964) found that ADH is synthesized on the ribosomes in the cell bodies of the supraoptic nuclei by following the incorporation of radioactive amino acids. Since neurosecretory granules were found in the cell body he proposed that the granules were synthesized in the Golgi bodies and then moved to the nerve terminals by axoplasmic streaming. Since a lag period was found between the formation of labeled protein and the formation of labeled vasopressin (Takabatake and Sachs, 1964) it is possible that ADH occurs in a precursor form before it is activated. There is no information as to whether the activation takes place in the cytoplasm or in the neurosecretory granules.

There is some information that the rate of synthesis can be controlled by physiological stimuli. During dehydration an increase in the vasopressin content of rat pituitaries was found even though the plasma

and urinary levels of vasopressin were increased (Ames and Van Dyke, 1950; Dicker and Nunn, 1957). It would seem a reasonable assumption that in most hormone systems increased synthesis would accompany increased release. This would be the only way a new level of release could be maintained for any period of time.

Most of the vasopressin in the neurohypophysis is located in the neurosecretory granules in the terminal nerve swellings (see Ginsburg, 1968, for review). The vasopressin is in combination with the protein neurophysin (Ginsburg and Ireland, 1966), a hormone binding carrier. The pH and enzyme-substrate concentration ratios are such that the hormone in the granules is mostly protein bound, whereas in the cytoplasm a larger percentage of the hormone is free.

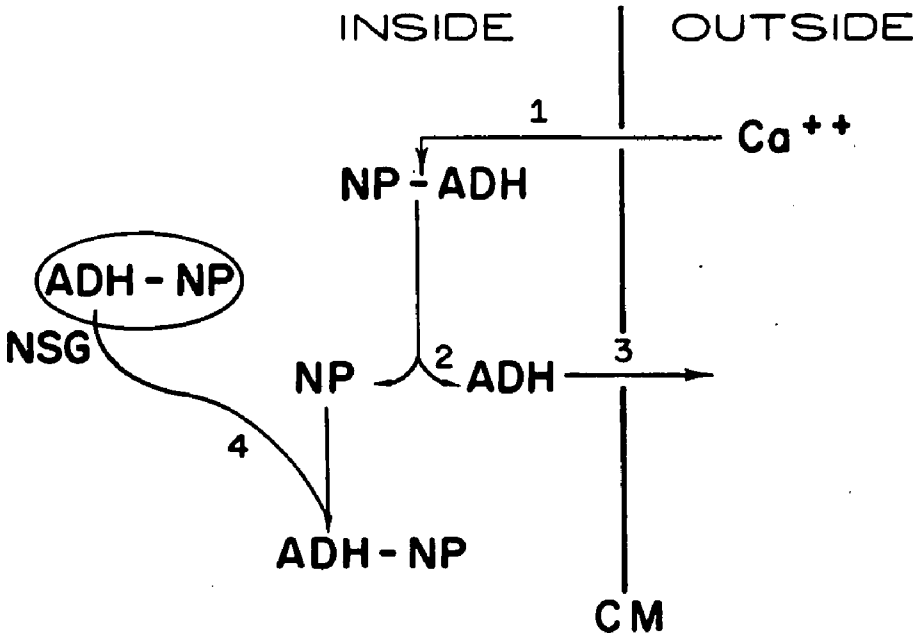
Most stimuli eliciting the release of ADH from nerve terminals probably act via synaptic input to the cell body and not directly on the releasing portion within the nerve terminal. For instance, Douglas and Poisner (1964) showed that an osmotic stimulus was not effective in releasing ADH from an isolated neurohypophysis although it is a very effective stimulus in vivo. The most likely candidate for the link between an input to the cell body and hormonal release from the nerve terminals would be electrical conduction down the cell membrane of the axon. Thus, it has been shown (Harris, 1947) that electrical stimulation of the pituitary leads to an increased release of hormone. Electrical stimulation of the isolated pituitary also leads to an increased release of ADH (Douglas and Poisner, 1964). Kandel (1962) made intracellular recordings from the hypothalmic portions of neurosecretory cells and showed that they conduct action potentials and exhibit excitatory post-synaptic potentials and inhibitory post-synaptic potentials. Finally, Ishikawa et al. (1966) showed changes in extracellular potentials in the pituitary stalk during stimulation of the supraoptic nuclei.

Once it has been established that electrical conduction along the neurosecretory cell is the link between input to the cell body and hormone release from the nerve terminal, one can look for a link

between the depolarization of the cell membrane and the release of hormone.

It has been proposed by Douglas and Poisner (1964) that calcium acts as the link between membrane depolarization and hormone release in a manner somewhat analagous to the situation in muscle. They found that a depolarization produced by raising the potassium concentration in the incubation medium of isolated pituitaries caused an increased release of ADH. If calcium was absent from the incubation medium there was no release of ADH. A dose response relation to the amount of calcium in the incubation medium was obtained. They also found that increasing the magnesium content in the incubation medium antagonized the response to calcium. These studies were then extended by observing the movement of radioactive calcium into the stalk. They observed an increased influx of calcium during potassium depolarization. Efflux was also increased but only when calcium was in the incubation medium. Thus, they felt that increased efflux was secondary to increased influx. The fact that metabolic inhibiters can prevent the release of ADH in the presence of calcium led to the suggestion that metabolism is involved in the response to calcium (Douglas et al., 1965). The fact that ATP did not lead to an increased release of ADH in vitro led Thorn (1966) to propose that calcium acts by inhibiting the binding of vasopressin to neurophysin. Thorn showed that the addition of calcium to the medium containing vasopressin inhibited the shift in elution fraction from columns which normally occurs when neurophysin is added (Smith and Thorn, 1965). Ginsburg and Ireland (1966) showed that suspensions of neurosecretory granules do not lose hormone in the presence of calcium.

The mechanism by which calcium might cause the release of ADH is shown in Figure 2. Upon depolarization there would be an increased entry of calcium into neurosecretory cells. The calcium would interfere with the binding of hormone to neurophysin in the cytoplasm and thus increase the concentration of free hormone. This would



CM - Cell membrane
ADH- Antiduretic hormone
NP - Neurophysin
NSG- Neurosecretory granule

Figure 2 -- Proposed mechanism by which calcium causes the release of ADH.

in turn increase the rate of movement of ADH out of the cell. Presumably, equilibrium would be reestablished by the movement of hormone from the neurosecretory granules into the cell water.

Stimuli for Release

Since the release of ADH occurs in an electrically active cell, we would like to know what are the inputs on the supraoptic nucleus that cause the neuroendocrine cells to fire. At the present time both cholinergic and adrenergic neurotransmitters are implicated. Pickford and Watt (1951) showed that acetylcholine will cause a reduction in the urine flow of a dog. The acetylcholine was effective at lower doses when injected in the carotid artery than when injected in a vein. The response to acetylcholine was not observed in dogs whose pituitaries had been removed. In addition the dogs discussed above had received atropine which should block systemic parasympathetic responses to acetylcholine.

It has also been shown that di-isopropyl-phosphofluoridate, an agent that binds cholinesterase, causes an inhibition of urine flow when injected in the supraoptic nucleus (Duke, Pickford, and Watt, 1950). Since the changes can occur with a denervated kidney, the authors conclude that acetylcholine acts on the supraoptic nucleus as a neurotransmitter. In addition, Daniel and Lederis (1966) have shown that acetylcholine causes the release of vasopressin when they remove the hypothalamus and neurohypophysis intact from a rat, whereas the incubated pituitary alone (Douglas and Poisner, 1964) does not release vasopressin in response to acetylcholine. Abrahams, Koele, and Smart (1957), using histochemical techniques, demonstrated the presence of cholinesterases in the cell bodies of the supraoptic nucleus. They felt that this indicated that acetylcholine could act as the neurotransmitter to activate the supraoptic cell, or that acetylcholine acts within the neuroendocrine cell as a type of internal neurotransmitter. The finding of Douglas and Poisner (1964) that acetylcholine does not cause a release

of vasopressin from the isolated neurohypophysis would seem to rule out the later interpretation. It would seem safe to conclude from the experiments on acetylcholine, that it is involved as a neurotransmitter and very likely that it is one of the neurotransmitters that activates the neuroendocrine cells.

The information with respect to epinephrine is much more confusing. O'Conner and Verney (1945) demonstrated that epinephrine blocked the drop in urine flow found after emotional stress. The drop in urine flow had a short and long term component. The long term component could be mimicked by the injection of posterior pituitary extracts into non-stressed dogs. Epinephrine blocked this long term effect in stressed dogs. When epinephrine and posterior pituitary extract were given at the same time, the long term drop in urine flow was still seen. From this they concluded that epinephrine acts in some manner to block the release of ADH from the pituitary and does not act by antagonizing the action of ADH on the kidney.

Dearborn and Lasagna (1952) found, on the other hand, that epinephrine in high doses can cause both a short and long term reduction in urine flow. The long term reduction was eliminated by removal of the posterior lobe. Also, Fang, Lui, and Wang (1962) found that electrical stimulation of many areas of the hypothalmus produced antidiuresis and that the response could be blocked by adrenergic blocking agents such as dibenzyline.

The central role of epinephrine is further complicated by the finding of Duke and Pickford (1951) that epinephrine sometimes inhibits the release of ADH produced by acetycholine and sometimes augments the release. The finding of Carlsson, Falck, and Hillard (1962), using fluorescent staining techniques, that the supraoptic nucleus is invervated by adrenergic fibers certainly would confirm the fact that epinephrine is involved in the central mechanism of ADH release. However, it is still unclear whether epinephrine serves to inhibit or stimulate the release of ADH. It is very possible it does both depending on the circumstance.

Assuming that hormones act to stabilize the internal environment, one would like to know what variations in the internal environment elicit a change in the circulating levels of ADH. Since ADH acts to conserve water it would seem reasonable to imagine that the stimulus for the release of ADH would be some parameter of water balance. The fact that animals with diabetes insipidus cannot conserve water and that desert animals which excrete a small amount of hypertonic urine have large amounts of ADH, are evidence for the water-conserving function of ADH. More direct evidence comes from the observations of Little and Radford (1964) in rats and Bonjour and Malvin (in press) in dogs that the plasma level of ADH increases after a twenty-four hour water deprivation.

Chambers, Melville, Hare, and Hare proposed in 1945 the theory that a change in osmolality of plasma is the physiological variable that determines the rate of ADH secretion. They showed that dogs with diabetes insipidus show a greater increase in urine volume than normal dogs during an infusion of hypertonic saline. The dogs with diabetes insipidus behaved like normal dogs when they were given pituitrin. They also showed that the diuresis produced by hypotonic saline infusions in normal dogs could be reduced by giving pituitrin but that no reduction in the diuresis produced by hypertonic saline could be achieved by giving pituitrin. They interpreted this finding to mean that ADH secretion was blocked by infusion of hypotonic saline and that ADH secretion was at maximal levels during infusion of hypertonic saline.

The theory that plasma osmolality controls ADH was greatly elaborated by the now classic work of Verney (1947) which showed that the dose of hypertonic saline necessary to produce an antidiuresis in dogs was considerably lower when administered in the carotid artery than when administered intravenously. Verney found that the response to hypertonic saline could be eliminated in animals in which the pituitary had been removed and could be matched by infusions of pituitary extract in normal dogs. A linear relationship between the increase of

osmotic pressure of plasma and the amount of pituitary extract needed to duplicate the response was observed. Thus, Verney concluded that the secretion of ADH was dependent upon the osmotic pressure of the plasma. An attempt was then made to further locate the osmoreceptors in the head. Denervation of the carotid sinus or carotid body did not eliminate the response. However, ligation of the internal carotid eliminated the response to hypertonic saline injected on that side. From this Verney concluded that the osmoreceptors were located somewhere in the area supplied by the internal carotid.

In later work (Jewell and Verney, 1957) it was found that antidiuresis occurred only when hypertonic saline reached an area in the anterior hypothalamus which includes the supraoptic nucleus. The work of Verney was substantiated in unanesthetized rats by Friedman, Hinke, and Friedman (1956) who found that a 1.5% saline solution produced an antidiuretic response. This response was not found in hypophysectomized animals or when the saline was injected into a vein rather than an artery. The finding of Woods and Bard (1960) that cats with a midbrain decerebration, leaving the hypothalamus and pituitary intact, showed a decreased urine flow with hypertonic saline is further indication that the osmoreceptors are located in the hypothalamus.

Some investigators feel that changes in tonicity might affect nerve cells in general rather than specific osmoreceptors. Thus, Reed and Vick (1961) showed that hypertonic saline mimics the general cardiovascular effects of acetycholine and that the responses are not eliminated by atropine. A facilitation of monosynaptic discharge in rats was found when hypertonic saline was injected in the carotid artery (Wagner, Grimsley, Kahan, and Stoller, 1963). These experiments certainly do not show the absence of specific osmoreceptors but rather indicate that tonicity affects nerve cells.

The fact that twenty-four hour dehydration in dogs produces about a two percent increase in osmolality (Wolf, 1957) is further indication that osmoreceptors are involved as the normal physiological

stimulus for ADH secretion. The only demonstration of the effects of overhydration is the observation of Anderson and Jewell (1957) of an increase in neurosecretory material in the neurohypophysis.

It is possible that ADH responds to changes in the fluid state of an animal through mechanisms that detect changes in blood volume. Rydin and Verney (1938) found that removal of small amounts of blood produced an antidiuresis that could not be eliminated by denervation of the kidney. Other observers were not able to duplicate this response (see Ginsburg, 1968, for review) but found that very large amounts of blood had to be removed to increase the level of ADH. Ginsburg and Heller (1953) showed that the antidiuretic activity of blood taken from rats increased with the amount of blood withdrawn. This was not found in hypophysectomized rats. Share (1962), using a method for ADH extraction and bioassay, demonstrated that decreasing the extracellular volume by peritoneal lavage with a hypertonic solution produced an increase in plasma ADH. If he infused isotonic dextran intravenously the rise was considerably reduced. In searching for volume receptors Share and Levy (1962) investigated the aortic and carotid baroreceptors. Vagotomy produced an increase in ADH. If the carotids were occluded after vagotomy a further increase in ADH was observed. However, occlusions of the carotids without vagotomy produced no rise in ADH. These results were interpreted as evidence for the role of baroreceptors in determining the levels of ADH secretion. Gauer and Henry (1963) believe the volume receptors are located on the low pressure side of the system, particularly in the left atrium. They interpret experiments which alter the distribution of blood volume, such as negative pressure breathing, changing position, affecting the pulmonary circulation, and balloons in the left atrium, as evidence for the role of left atrial volume receptors. Thus, inflation of a balloon in the left atrium caused a diuresis which could be blocked by vagal cooling. This effect could not be explained by changes in renal function such as GFR.

A third possibility for the regulation in response to changes in

fluid balance is via the renin-angiotensin system. Thus, a decrease in the amount of sodium reaching the macula densa in the kidney leads to an increase in the release of renin (Vander, 1964). Renin in the plasma acts as an enzyme to split the peptide angiotensin from a precursor protein. Angiotensin then acts to simulate the release of a salt retaining hormone, aldosterone. Since aldosterone and ADH increase under similar conditions, Bonjour and Malvin (pers. comm., 1969) investigated the possibility that angiotensin plays a role in ADH release. They found that angiotensin caused an increase in ADH. This was true of an infusion of exogenous angiotensin or as a result of endogenous angiotensin formed by the infusion of renin. They found a dose response in the sense that the magnitude of the increase in mean arterial pressure was correlated with the size of the increase in plasma ADH. Since carotid infusions were effective at a lower dose than venous infusions, they felt that the receptor was located in the head. Ventricular-cisternal brain profusions of angiotensin also led to an increase in plasma ADH.

It seems clear that volume receptors, osmoreceptors, and the renin-angiotensin system all play a role in the regulation of ADH. Since the blood volume must be reduced considerably before one gets an increase in ADH, volume receptors would not seem to play a major role in the day to day regulation of ADH. A twenty-four hour dehydration produces almost no drop in blood volume but does produce about a two percent increase in plasma osmolality. This is sufficient to raise the plasma ADH concentration. Thus, osmolality would seem to be more important than volume in the response to dehydration.

Thesis Problem: Effects of Calcium on ADH

For my thesis I have attempted to extend the information indicating a link between calcium and the release of ADH from the posterior pituitary. Calcium has been shown to be involved in the release of a number of hormones. Douglas (1966) showed that the response of the isolated, perfused adrenal gland to acetylcholine depends on the amount of

calcium in the perfusate. This is associated with an increased uptake of calcium. Since the same phenomenon is observed in the potassium stimulated adrenal medulla, Douglas feels that calcium is probably a link in the release of epinephrine from the adrenal medulla. Burn and Gibbon (1965) demonstrated that calcium is necessary in the medium to observe norepinephrine release from sympathetic fibers supplying the rat ileum and isolated rabbit atria. In addition, it has been observed (Dodge and Rahamimoff, 1967) that acetylcholine release from isolated nerve terminals shows a dose response relationship with the amount of calcium in the bathing medium. Similar experiments have implicated calcium in the release of insulin (Curry, Bennett, and Grodsky, 1968) and prolactin (Parsons, 1969).

Douglas and Poisner (1964) were the first to show that calcium played a role in the release of ADH from the posterior pituitary gland. They demonstrated that the isolated pituitary, when depolarized, will release ADH in a dose-response relationship to the amount of calcium in the bathing medium. The release of ADH is associated with the uptake of calcium by the cell. The finding (Smith and Thorn, 1965; Ginsburg, Jayasene, and Thomas, 1966) that calcium interferes with the binding of ADH to neurophysin led to the theory, illustrated in Figure 2, that upon depolarization calcium enters the neurosecretory cell and interferes with the binding of hormone to neurophysin in the cytoplasm. The higher concentration of hormone would then cause a greater movement of hormone from the inside to the outside of the cell. Equilibrium would be established by the movement of hormone from the neurosecretory granules to the cytoplasm.

In my experiments I attempted to show that the relationship between calcium and ADH which Douglas and Poisner observed <u>in vitro</u> also took place in the intact animal. Calcium was infused into anesthetized dogs and plasma ADH levels were measured using a method involving extraction and partial purification of ADH from plasma. Indirect evidence already exists that calcium causes a release of ADH in

the intact animal. The infusion of calcium into normal rats (Thorn, Smith, and Skadhauge, 1965) produced an antidiuresis. Calcium infused in the carotid artery was more effective than when infused intravenously, an indication that calcium was acting in the head. Calcium did not produce an antidiuresis in rats with diabetes insipidus, implicating ADH in the response to calcium. Dyball (1968) infused calcium into rats and measured the ADH in blood using a bioassay. He found that calcium increased plasma ADH when infused in a vein. Lesnik, Gusek, and Truczyk (1969) performed experiments on rats in which they removed the jaw and exposed the pituitary. They then took out the stalk and dripped isotonic saline over the exposed stalk. The wash was collected and analyzed for ADH. They found that an infusion of calcium produced an increase in the antidiuretic activity of the wash.

The experiments cited above indicate that the relationship between calcium and ADH probably exists in vivo as well as in vitro. However, they are all subject to rather serious criticism. Thorn's experiments do not involve any measurements of plasma ADH or renal function. Thus, it cannot be certain that the antidiuresis he observed was due to a change in ADH. In Dyball's experiments he did not use any final control after the period of calcium infusion. He took two ml of blood for his initial control and then two more ml after infusing calcium. In a rat the removal of four ml of blood represents a substantial hemorrhage. In addition, he used whole blood and plasma in his bioassay. This leaves the possibility that he was measuring something other than ADH in his bioassay. The experiments of Lesnik et al. also did not involve a final control period or any purification before injection in the bioassay animal. In addition their animals are not really intact.

In my experiments I also compared carotid infusions of calcium to venous infusions of calcium. In addition, experiments were done using ventriculo-cisternal brain perfusions. It is believed that changes in calcium in this system would not reach the posterior pituitary but would affect areas in the hypothalamus.

In order to see if my ventriculo-cisternal brain perfusion system was working I perfused hypertonic saline in two experiments. If the hypertonic saline produced an increase in ADH I could be sure that the brain perfusion was reaching at least some structure capable of eliciting an increase in plasma ADH.

The use of dogs in my experiments rather than rats enabled me to take large amounts of blood without producing a significant hemorrhage. In all cases a final control blood sample was taken. If the ADH level in this sample was considerably different from the initial control the experiment was discarded.

In addition to experiments in which plasma calcium was raised, I injected thyrocalcitonin in a series of experiments in an attempt to lower plasma calcium. Douglas and Poisner (1969) found that magnesium inhibited the release of ADH in vitro. I performed a series of experiments to see if this effect could also be observed in vivo.

There is one other problem I hoped to answer in my experiments. Hypercalcemia has long been associated clinically with a loss of concentrating ability in the kidneys. Manitius, Levitin, and Epstein (1960) associated the defect with a decrease in the cortico-papillary concentration gradient. However, they found the decrease in urine osmolality greater than the fall of solute gradient in the papilla. They interpreted this to mean a decrease in the water permeability of the collecting duct in addition to a decrease in sodium reabsorption. Gill and Bartter (1961) demonstrated that the loss of concentrating ability in human hypercalcemic states is independent of the disease that raises the calcium. They felt this showed that the loss was a direct effect of calcium on the kidney. Suki, Eknoyan, Rector, and Seldin (1969) found that the clearance of free water and the reabsorption of free water were both reduced in hypercalcemic dogs. They felt that this indicated that the loss of concentrating ability was due to a failure of sodium reabsorption in the ascending limb rather than a decrease in the permeability of the collecting duct. The understanding of the counter current system is not

really advanced enough at the present time to permit an interpretation of changes in urine concentration. If calcium raises the level of plasma ADH in the intact animal it seems clear that this loss of concentrating ability is not brought about by insufficient release of ADH and that one must look elsewhere to explain this phenomenon.

METHODS

Experimental Procedure

Experiments were performed on twenty-six mongrel dogs weighing 17 to 34 kilograms. Dogs were maintained on Friskies dry dog food until the night before an experiment was performed. The dogs were fasted overnight before experiments but allowed free access to water until the time of anesthesia. Two ml of Innovar^{R} were injected intramuscularly as a pre-anesthetic and then anesthesia was achieved by an intravenous injection of sodium pentobarbitol, 20 mg/kg. In all but the preliminary experiments a tracheotomy was performed and respiration maintained by a Harvard Respiration Pump. A polyethylene catheter was inserted in the left femoral artery for the purpose of withdrawing blood and was connected by a three-way stopcock to a mercury mannometer for recording blood pressure. Other surgical procedures depended on the particular experiment and will be described accordingly. Blood samples were obtained by allowing approximately 60 ml of blood to flow from the artery into a plastic centrifuge tube containing heparin. The blood was centrifuged at 2,200 g for 10 minutes and the plasma removed with a Pasteur pipette. A 4 ml aliquot of plasma was taken for calcium, magnesium, sodium, and osmolality analysis. The rest was used for ADH analysis. A 0.9% sodium chloride solution of 6% dextran 70 was added to the red blood cells to a volume of 50 ml. These red blood cells in dextran were given back to the dog immediately after the next blood sample was taken. Since there were no red cells available, the first blood sample was followed immediately by the administration of 50 ml of 6% dextran 70 in 0.9% sodium chloride.

I did six preliminary experiments to determine the proper time periods and doses of calcium that would reveal the effects of calcium on antidiuretic hormone. In the first two experiments calcium was infused in the carotid artery; in the second two experiments calcium was infused in the vertebral artery, and in the last two experiments calcium was

infused during different periods in the carotid artery and femoral vein.

In the carotid experiments a twenty-three gauge needle was inserted in the right carotid artery. One hour after surgery was completed a control blood sample was taken. During this hour an infusion of 0.9% saline was delivered through the arterial needle at 0.15 ml/min. After the control sample was obtained an infusion of calcium chloride was delivered through the carotid arterial needle. In one dog a solution of 60 mM $CaCl_2$, 88 mM $NaCl_2$ was infused at 0.8 ml/min. Half an hour after the infusion of calcium chloride had begun a second blood sample was taken. The infusion was continued for another half hour and a third blood sample was taken. The calcium chloride infusion was then stopped and the infusion of isotonic saline resumed. One hour later a final control blood sample was taken. The same procedure was done on a second dog except that 120 mM $CaCl_2$ was infused at 1.9 ml/min.

In order to determine if calcium was more effective when infused in the vertebral artery than in the carotid artery, a twenty-three gauge needle was inserted in the vertebral artery in two dogs. In one dog 60 mM $CaCl_2$ was infused at 0.8 ml/min. In the other dog 120 mM $CaCl_2$ was infused at 1.9 ml/min. The time periods were the same as during the carotid infusions.

In a fifth preliminary experiment a comparison was made between a carotid and venous infusion of calcium chloride. In this dog a twenty-three gauge needle was inserted in the carotid artery and a polyethylene catheter in the femoral vein. The carotid needle was kept open during the control period by an infusion of 0.9% saline at 0.15 ml/min. One hour after surgery was completed a control blood sample was taken. Calcium chloride, 120 mM, was then infused into the carotid artery at 1.91 ml/min. for thirty minutes. A second blood sample was taken and then the infusion of calcium chloride was stopped. After waiting an hour a control blood sample was taken. Calcium chloride, 120 mM, was then infused at 1.91 ml/min into the femoral vein. After 30 minutes a fourth blood sample was taken and then the infusion stopped.

An hour later a final control sample was taken. In order to see if there is a difference in response between the two carotid arteries the above experiment was repeated with an additional experimental period involving the infusion of calcium chloride in the left carotid artery.

The preliminary experiments indicated that calcium chloride might increase levels of plasma ADH and that calcium chloride might be more effective when infused in the carotid artery than when infused in the femoral vein. Since the dogs were not able to maintain stable control values of ADH over long periods of time I decided to test the difference between the effects of carotid and of venous infusions of calcium in different dogs.

Experiments to test the effects of venous infusions of calcium chloride were performed on four dogs. A tracheotomy and the placement of catheters in the femoral artery and vein were the only surgery performed. An infusion of 0.9% saline at 0.15 ml/min was then begun into the femoral vein. An hour after surgery the first control blood sample was taken. The control saline infusion was then changed to an infusion of 110 mM $CaCl_2$ at 1.15 ml/min. Twenty minutes after the infusion of $CaCl_2$ was begun a second blood sample was taken. The rate of infusion was then increased to 1.91 ml/min. After another twenty minutes a third blood sample was taken. The $CaCl_2$ infusion was then stopped and the saline infusion resumed. An hour after the end of the $CaCl_2$ infusion the final control blood sample was taken.

In four other dogs a twenty-three gauge needle was placed in the carotid artery in addition to the femoral arterial and venous catheters. A protocol identical to the one described for venous infusions of $CaCl_2$ was followed, except that the saline and $CaCl_2$ solutions were infused into the carotid artery instead of the femoral vein.

In an attempt to localize further the area of the brain that is sensitive to calcium, ventriculo-cisternal brain perfusion was done in three of the dogs. The method used was based on the one developed by Papenheimer (1962). After the placement of catheters in the femoral

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vein and artery and the performance of a tracheotomy, the head of the dog was placed in a stereotaxic apparatus. Using an electrode carrier, a ventricular cannula was placed through a trephined hole in the cranium. The cisternal cannula was placed through a small skin incision and the cannula directed through the muscles on the posterior surface of the cranium until it punctured the cisterna magna. The ventricular cannula was a 21 gauge stainless steel tube, and an 18 gauge stainless steel tube was used for the cisternal cannula. A three-way connector was placed just in front of the inflow needle for the continuous measurement of inflow pressure. Placement of the cannula was determined by changes in inflow pressure and stereotaxic measurement.

An artificial cerebrospinal fluid used as control was made up as follows: sodium, 151 mM/L; magnesium, 0.8 mM/L; phosphate, 0.5 mM/L; calcium, 1.14 mM/L; and bicarbonate, 25.0 mM/L. Solutions of cerebrospinal fluid containing higher concentrations of calcium were made by adding calcium to control cerebrospinal fluid. The "low" calcium solution contained 1.43 mM/L calcium. The "high" calcium solution contained 1.60 mM/L calcium. Hypertonic saline was infused during one experimental period to see if the system was working. The hypertonic saline was made up by adding 25 mM/L NaCl to the control cerebrospinal fluid so that it contained 176 mM/L sodium and 157 mM/L chloride. All solutions were gassed with 5% CO_2 and 95% O_2 at 38° before being delivered to the animal. Solutions were delivered at 0.6 ml/min by a Harvard infusion pump. Control cerebrospinal fluid was perfused for one hour during control periods and the different experimental cerebrospinal fluids were infused for one-half hour during experimental periods.

Thyrocalcitonin was administered to five dogs in order to test the effects of lowering plasma calcium on the level of plasma ADH. One hour after a traceostomy and the placing of catheters in the femoral artery and vein, a control blood sample was taken. Porcine thyrocalcitonin, 176 MRC units in 5 ml of 0.9% saline, brought to pH 4 by the

addition of a drop of acetic acid, was then administered intravenously. Blood samples were taken one-half hour, one hour, and two hours after the thyrocalcitonin was given.

Since the in vitro studies had shown an inhibitory effect of magnesium on ADH release, I tested the effect of $MgCl_2$ infusion in the carotid artery in five dogs. The protocol was the same as that used for the infusion of calcium chloride in the carotid artery, except that 110 mM $MgCl_2$ was infused.

Methods of Analysis

ADH Analysis was done by the method developed by Weinstein, Berne, and Sachs (1960) and modified by Share (1961), Share and Levy (1966), and Bonjour and Malvin (in press). Four volumes of 12.5% trichloroacetic acid at $4^{\circ}C$ were added to 22 to 30 ml of plasma. The mixture was shaken and centrifuged at 2,000 g for six minutes. The supernatant was decanted and saved and the precipitate washed with two volumes of 10% TCA. This was centrifuged again at 2,000 g for six minutes and the supernatant pooled with the one from the first spinning. The supernatants were then frozen.

The next step in purification involved column chromatography. Glass columns, 2 cm in diameter, were packed with resin (Amberlite CG-50, Rohm and Hass, Philadelphia) 2 cm high. The resin was prepared by the method of Hirs, Moore, and Stein (1953). The TCA supernatant was thawed and added to the columns in the cold room. Rate of flow was adjusted to 2 ml/min by a clamp on a piece of rubber hose at the bottom of the column. After the TCA supernatant had passed through the columns, 25 ml of glass-distilled water was passed through as a wash. The ADH was then removed from the columns with 25 cc of a pyridine solution (30% pyridine, 4% acetic acid by volume). The eluate was collected in flasks containing 3 mg L - cystine in .5 ml of 17% formic acid. The following day the eluate was evaporated to dryness in a vacuum with a rotating evaporator. The flasks were in a

water bath maintained at 30°C. After drying, the residue was taken up in 2 ml of glass-distilled water and evaporated to dryness again. The residue was washed with water three times. Samples were prepared for bioassay by being dissolved in a solution of 0.9% NaCl and 0.03% acetic acid, and centrifuged to remove the L - cystine. The samples were then kept frozen until the bioassay.

Bioassay was performed on 130 to 170 gram male rats that had been fasted and deprived of water for 18 to 24 hours before the bioassay. In some of the later experiments the rats were allowed free access to food and water until the time of the bioassay. The rats were hydrated and anesthetized with 12% ethanol given in two doses, the total ethanol equal to 3% of the body weight. The doses were given approximately thirty minutes apart. Additional doses were given approximately thirty minutes apart if needed to achieve anesthesia. A tracheostomy was then performed and the trachea cannulated with PE 240 tubing. The external jugular vein was isolated and cannulated with PE 50 tubing. A priming dose of infusion solution (3% dextrose, 0.19% NaCl, and 2% ethanol) was administered and the solution was then infused into the vein by a Harvard infusion pump at .15 ml/min. The urethra was ligated and the bladder catheterized with PE 200 tubing. Urine conductivity was measured by having the urine flow through two hollow electrodes separated by 1 cm of polyethylene tubing. The voltage drop between the two electrodes was recorded on a Grass polygraph with the area under the conductivity curve taken as the index of response to a given dose of ADH. All unknown samples were injected at least three times in a minimum of two different rats. The injections of unknown were bracketed by injections of USP Posterior Pituitary Reference Standard. Volumes of injection varied between 0.1 and .25 ml and the catheters were rinsed with 0.05 ml (maximum) of infusion solution. The average results from at least three injections of unknown was taken as the ADH level of a particular sample. Previous experiments showed a recovery of 85% $\pm$ S.E. 2.3% using this method.

Arterial Pressure - Arterial pressure was recorded using a mercury manometer.

Sodium - The concentration of sodium in plasma was determined by flame photometry.

Osmolality - Osmolality of plasma was measured by freezing point depression in a Fisk Osmometer.

Calcium - The concentration of calcium in plasma was measured by Atomic Absorption Spectrophotometry.

Magnesium - The concentration of magnesium in plasma was measured by Atomic Absorption Spectrophotometry.

RESULTS

Preliminary experiments on the effects of $CaCl_2$ infusions on plasma levels of ADH

Table 1 gives the data from six preliminary experiments. In dogs 1 and 2 $CaCl_2$ was infused in the carotid artery. It can be seen that the lower dose, 60 mM $CaCl_2$ in 88 mM NaCl at 0.7 ml/min, given in dog 1 causes only a slight rise in plasma ADH, whereas the higher dose, 120 mM $CaCl_2$ at 1.9 ml/min, given in dog 2 caused a substantial increase in plasma ADH after $\frac{1}{2}$ hour. Previous experiments in our laboratory (Bonjour and Malvin, in press) have indicated that the recovery of ADH added to plasma averaged 85% $\pm$ S. E. 2.3%. This would indicate that our method can detect about a 20 percent difference in ADH levels of individual samples of plasma.

In dogs 3 and 4 $CaCl_2$ was infused in the vertebral artery. Dog 3 received the same dose of $CaCl_2$, 60 mM $CaCl_2$ in 88 mM NaCl at 0.7 ml/min, as dog 1. There was no increase in plasma ADH during the $CaCl_2$ infusion. Dog 4 received the same dose of $CaCl_2$, 120 mM $CaCl_2$ at 1.9 ml/min, as dog 2. This dose produced an increase in plasma ADH. All of the ADH values in dog 4 are considerably higher than in the first three dogs. Bonjour and Malvin (in press) have shown that the control value of plasma ADH is a function of surgical trauma. There was considerable bleeding during the surgery performed on this dog which probably accounts for the difference in control levels of ADH.

In dogs 5 and 6 an attempt was made to compare carotid and venous infusions of $CaCl_2$ in the same dog. In both dogs an infusion of 120 mM $CaCl_2$ at 1.9 ml/min into the carotid artery during the first experimental period caused a rise in ADH. It was impossible, however, to compare carotid artery and femoral vein infusions in the same dog because the ADH control level rose toward the end of the experiment. This same increase was seen in the final control period of dog 3 and will be discussed further in the discussion section.

Table 1: Preliminary experiments on the effects of calcium infusion on ADH.

Dog	Period	Route of Infusion	Calcium Concentration of infusate mM/L	Rate of Infusion ml/min	ADH µU/ml	Plasma Calcium mM/L	Mean Arterial Pressure mmHg
1	control				4.9	3.1	106
	½ hr $CaCl_2$	right carotid	60	.764	6.6	3.2	108
	1 hr $CaCl_2$	"	60	.764	5.5	3.3	104
	control				4.9	2.9	104
2	control				2.5	3.0	116
	½ hr $CaCl_2$	"	120	1.91	8.0	3.5	116
	1 hr $CaCl_2$	"	120	1.91	10.1	4.0	120
	control				2.6	3.2	114
3	control				7.3	2.9	98
	½ hr $CaCl_2$	right vertebral	120	.764	7.7	3.1	96
	½ hr $CaCl_2$	"	120	.764	7.9	3.5	96
	control				17.3	3.3	96
4	control				26	2.9	124
	½ hr $CaCl_2$	right carotid	120	1.91	43	3.2	120
	1 hr $CaCl_2$	"	120	1.91	60	3.9	120
	control				41	3.1	118

Table 1: Continued

Dog	Period	Route of Infusion	Calcium Concentration of Infusate mM/L	Rate of Infusion ml/min	ADH μU/ml	Plasma Calcium mM/L	Mean Arterial Pressure mmHg
5	control	right carotid	120	1.91	1.8	2.8	100
	½ hr $CaCl_2$				11.2	4.3	100
	control	femoral vein	120	1.91	4.2	3.7	104
	½ hr $CaCl_2$				5.3	4.4	104
	control				11.2	3.6	98
6	control	right carotid	120	1.91	1.6	2.9	126
	½ hr $CaCl_2$				3.3	3.9	124
	control	femoral vein	120	1.91	1.7	3.2	124
	½ hr $CaCl_2$				7.4	4.1	110
	control	left carotid	120	1.91	80	3.5	100
	½ hr $CaCl_2$				99	4.4	100
	control				15	3.6	118

The effect of femoral vein infusion of $CaCl_2$ on the plasma level of ADH

It can be seen from Figure 3 that the lower dose of $CaCl_2$, 120 mM at 1.15 ml/min, caused no significant increase in plasma ADH when infused into the femoral vein for 20 minutes ($p = .60$ using paired sample analysis). At the higher dose of $CaCl_2$, achieved by raising the rate of venous infusion to 1.9 ml/min for an additional 20 minutes, there was still no significant change ($p = .13$) in plasma ADH. Table 2 indicates that there was no change in plasma sodium, osmolality, or mean arterial pressure during these infusions that would cause a change in plasma ADH.

The effect of carotid artery infusion of $CaCl_2$ on the plasma level of ADH

Figure 3 shows that there was a substantial increase in plasma levels of ADH when $CaCl_2$ was infused into the carotid artery. The doses of $CaCl_2$ were the same as those used during the venous infusions. The response of plasma ADH to the lower dose of $CaCl_2$, 120 mM at 1.15 ml/min, were statistically significant at the $p = .02$ level. The response to the higher dose of $CaCl_2$, achieved by raising the rate of infusion into the carotid artery to 1.9 ml.min for an additional 20 minutes, was significant at the $p = .005$ level. Figure 4 shows that the rise in plasma calcium was the same during both carotid artery and femoral vein infusions.

The effects of ventriculo-cisternal brain perfusions of $CaCl_2$ and hypertonic saline on the plasma level of ADH

Figure 5 illustrates that raising the calcium concentration of the artificial cerebrospinal fluid perfusing the brain from 1.14 mM/L to a "low" dose of 1.43 mM/L or a "high" dose of 1.6 mM/L caused no change in the plasma level of ADH. The lower dose was a 25 percent increase in calcium concentration and the higher dose was a 40 percent increase in calcium concentration. This percentage change in calcium

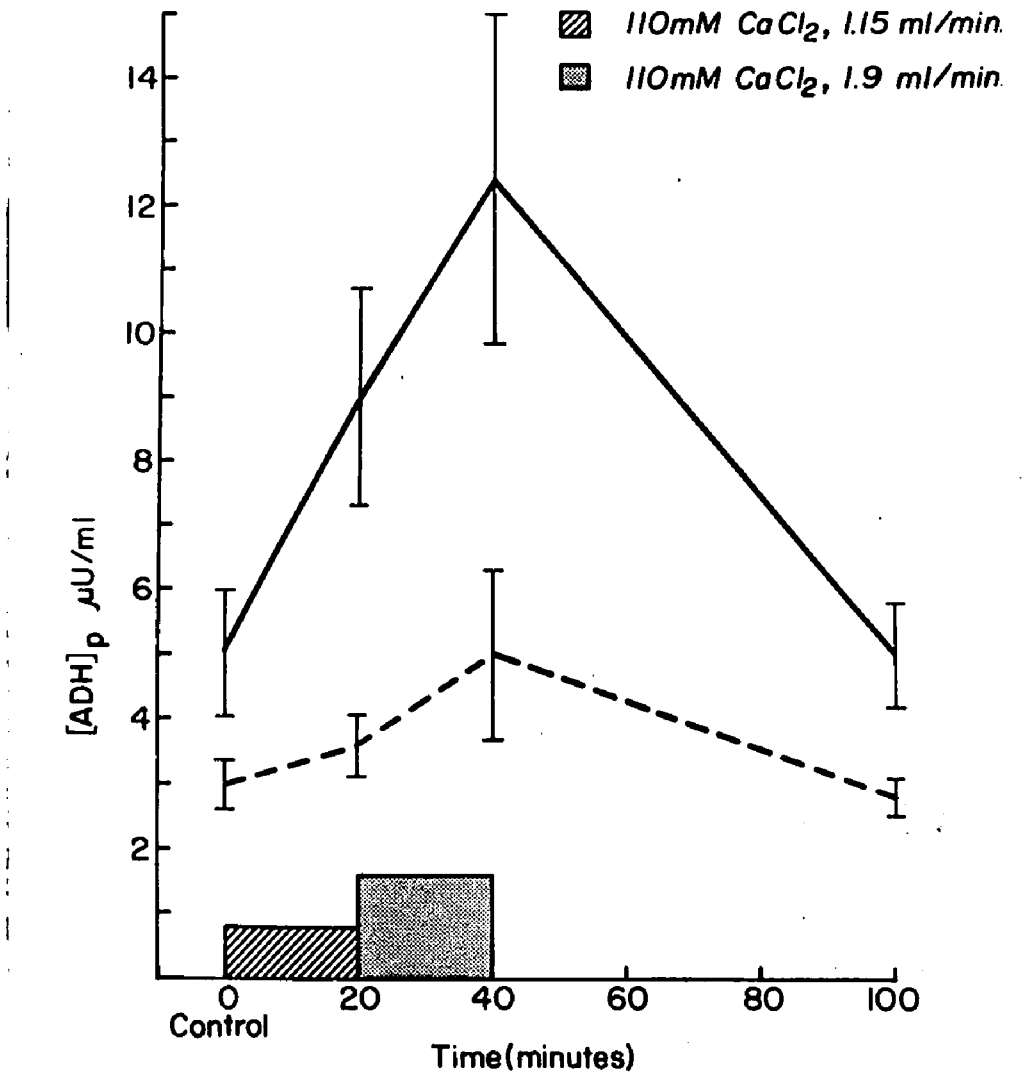


Figure 3 -- Effects of carotid artery and femoral vein infusions of calcium chloride on plasma levels of ADH. Each point represents the average ± S.E.M. of four experiments. Straight line represents carotid infusions; broken line represents venous infusions.

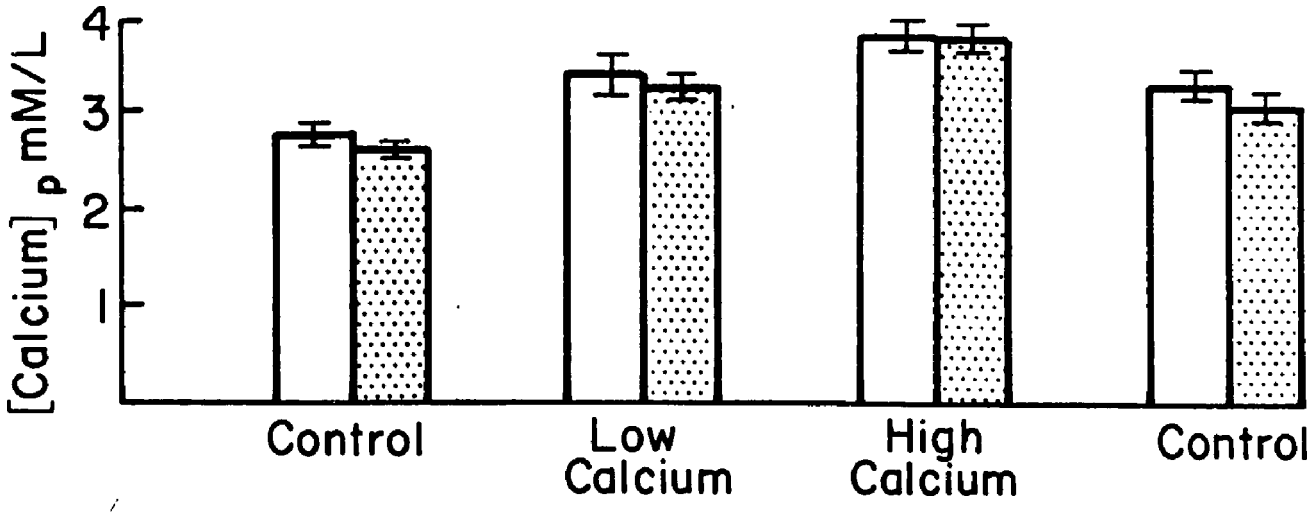


Figure 4 -- Changes in plasma calcium during calcium chloride infusions in the carotid artery and femoral vein. Each bar represents the average ± S.E.M. of four experiments. Blank bars are carotid experiments; stippled bars are venous infusions.

Table 2: Effects of venous infusion of calcium chloride on plasma levels of ADH, calcium, sodium, osmolality, and mean arterial pressure.

Dog	Infusion	Plasma ADH μU/ml	Plasma Calcium mM/L	Plasma Sodium mM/L	Plasma Osmolality mOsmol/$K_g H_2O$	Mean Arterial Pressure mm H_g
7	control	4.3	2.8	150	307	84
	low Ca+	3.4	3.0	149	305	89
	high Ca+	3.1	3.6	150	305	90
8	control	2.2	2.4	149	299	90
	low Ca+	3.3	3.2	147	297	92
	high Ca+	5.0	3.6	147	298	94
	control	2.6	3.0	145	296	94
9	control	2.3	2.6	140	292	84
	low Ca+	2.7	3.4	144	295	90
	high Ca+	3.0	3.8	149	299	92
	control	2.3	3.2	149	300	108
10	control	3.2	2.6	145	296	122
	low Ca+	5.2	3.2	146	295	126
	high Ca+	9.1	4.0	145	294	130
	control	3.6	2.8	147	297	126

Table 3: Effects of carotid artery infusion of calcium chloride on plasma levels of ADH, calcium, sodium, osmolality, and mean arterial pressure.

Dog	Infusion	Plasma ADH μU/ml	Plasma Calcium mM/L	Plasma Sodium mM/L	Plasma Osmolality mOsmol/$K_g H_2O$	Mean Arterial Pressure mm H_g
11	control	2.5	2.8	151	306	124
	low Ca+	6.3	3.8	150	303	130
	high Ca+	8.3	4.0	150	302	130
	control	3.0	3.4	150	302	120
12	control	5.0	2.8	147	303	120
	low Ca+	8.2	3.4	146	300	122
	high Ca+	12.4	3.8	150	300	128
	control	5.2	3.2	151		124
13	control	5.4	2.6	149	298	92
	low Ca+	14.6	3.2	149	298	90
	high Ca+	20.7	3.8	146	295	90
	control	5.3	3.4	145	293	94
14	control	8.0	2.6	146	300	96
	low Ca+	7.1	3.2	147	301	102
	high Ca+	8.4	3.6	145	301	100
	control	6.8	3.0	145	299	98

was the same as that observed during carotid artery and femoral vein infusions of $CaCl_2$. Figure 5 also shows that the perfusion of hypertonic saline, formed by adding an additional 25 mM/L NaCl to the control cerebrospinal fluid, through the ventricular system produced a marked increase in plasma ADH. It can be seen from Table 4 that there was no change in plasma sodium, plasma osmolality, or mean arterial pressure during the perfusions.

The effect of thyrocalcitonin injection on the plasma level of ADH

The administration of thyrocalcitonin was followed by a marked rise in plasma ADH. Figure 6 shows the changes in plasma ADH 30 minutes, 60 minutes, and 120 minutes after the administration of thyrocalcitonin. The plasma ADH was significantly different from control 60 minutes after the injection of thyrocalcitonin ($p = .02$). As can be seen from Table 5 the concentration of calcium decreased following the administration of thyrocalcitonin. Table 5 also shows that there was a substantial fall in the blood pressure over the same time period in all but dog 18. Dog 18, in which there was no decrease in plasma calcium after thyrocalcitonin, also had no decrease in blood pressure and no rise in plasma ADH.

The effect of carotid artery infusion of $MgCl_2$ on the plasma level of ADH

Infusion of 110 mM $MgCl_2$ produced no change in plasma ADH as can be seen in Figure 7. Table 6 shows that there were no changes in plasma sodium, plasma osmolality, or mean arterial pressure that would influence the plasma ADH.

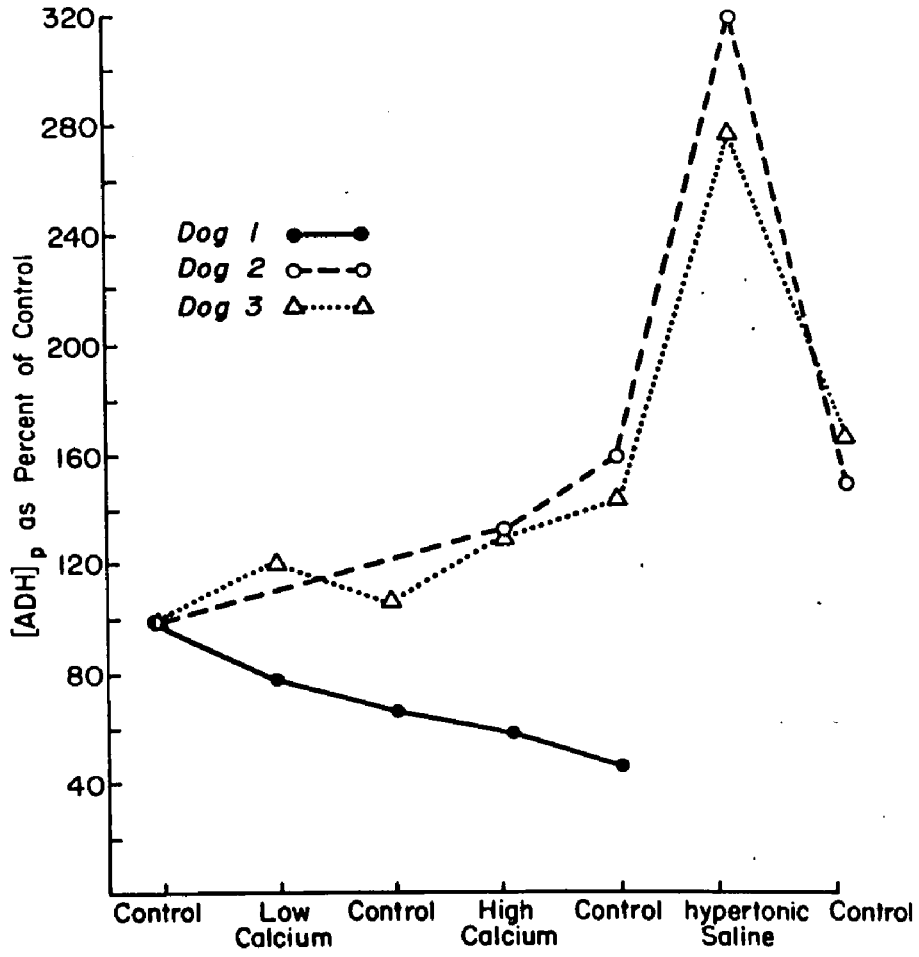


Figure 5 -- Effects of ventriculo-cisternal brain perfusions of calcium chloride and hypertonic saline on the plasma level of ADH.

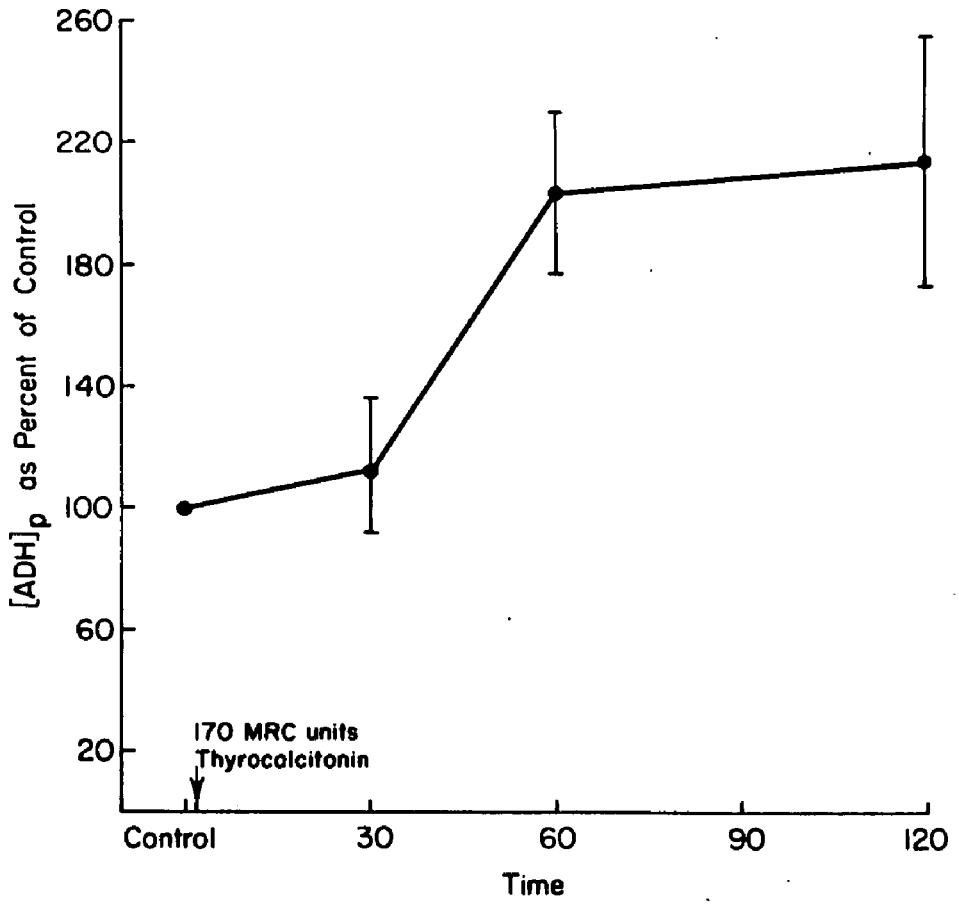


Figure 6 -- Effects of thyrocalcitonin on plasma level of ADH. Each point represents the average ± S.E.M. of four experiments.

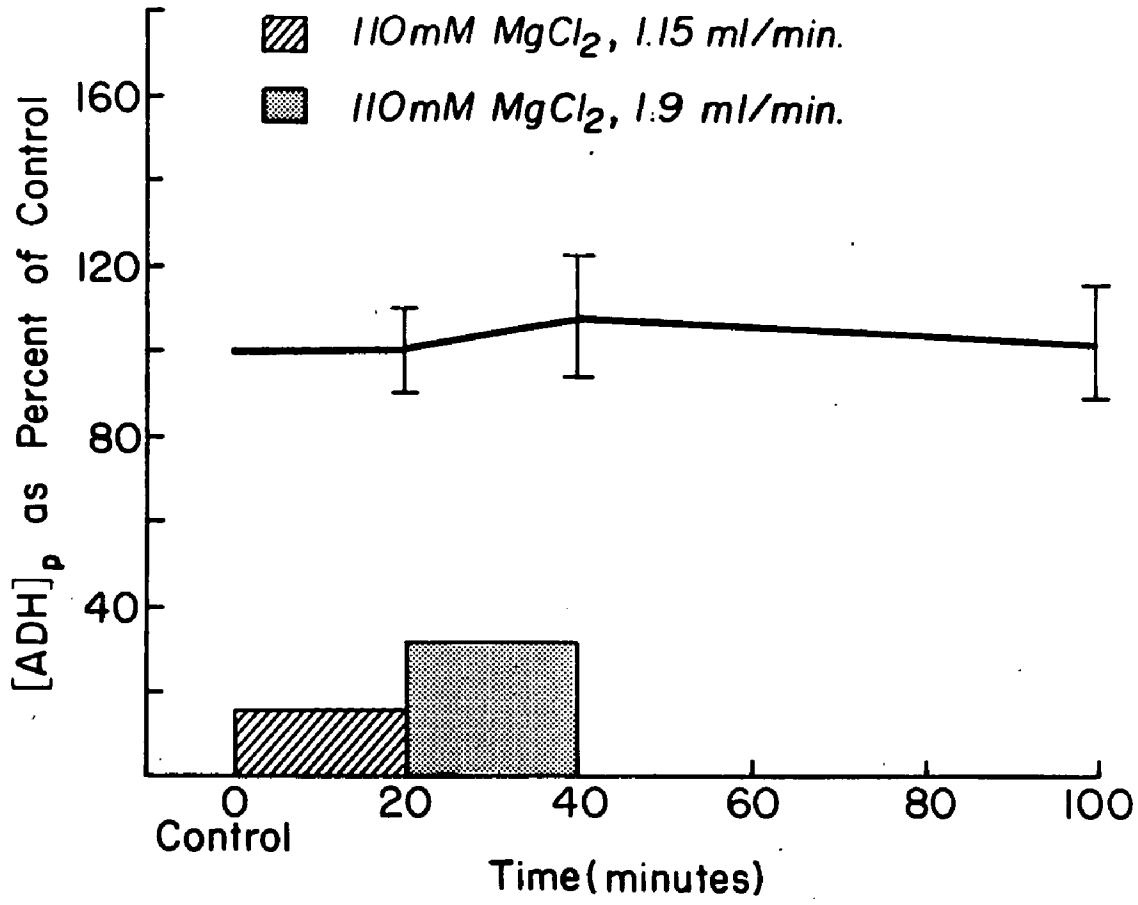


Figure 7 -- Effect of carotid artery infusion of magnesium chloride on the plasma level of ADH. Each point represents the average ± S.E.M. of five experiments.

Table 4: Effects of ventriculo-cisternal brain perfusions of calcium chloride and hypertonic saline on plasma levels of ADH, calcium, sodium, osmolality, and mean arterial pressure.

Dog	Period	Plasma ADH μU/ml	Plasma Calcium mM/L	Plasma Sodium mM/L	Plasma Osmolality mOsmol/$K_g H_2O$	Mean Arterial Pressure mm H_g
15	control	6.0	-	-	-	135
	low Ca++	4.6	2.5	146	300	140
	control	3.9	2.4	148	303	140
	high Ca++	3.4	2.5	146	298	140
	control	2.7	2.5	150	307	138
16	control	2.8	2.6	140	284	95
	low Ca++	3.4	2.6	140	286	100
	control	3.0	2.5	144	295	98
	high Ca++	3.6	2.5	138	281	92
	control	4.0	2.4	138	283	92
	hypertonic saline	7.8	2.4	144	293	96
	control	4.7	2.4	142	290	88
17	control	27.4	2.2		292	98
	high Ca++	35.8	2.2		280	96
	control	43.5	2.3		282	100
	hypertonic saline	87.5	2.2		279	98
	control	40.9	2.2		289	98

Table 5: Effects of thyrocalcitonin on plasma levels of ADH, calcium, sodium, osmolality, and mean arterial pressure.

Dog	Period	Plasma ADH μU/ml	Plasma Calcium mM/L	Plasma Sodium mM/L	Plasma Osmolality mOsmol/$K_g H_2O$	Mean arterial Pressure mm H_g
18	control	7.6	2.5	141	285	108
	30 minutes	6.8	2.3	141	287	112
	60 "	9.7	2.5	145	293	110
	120 "	6.7	2.5	141	286	112
19	control	26.7	2.5	142	289	110
	30 minutes	23.1	2.3	142	291	112
	60 "	46.9	1.9	148	297	100
	120 "	58.4	1.9	150	304	100
20	control	4.1	2.8	140	287	114
	30 minutes	7.7	2.5	142	291	110
	60 "	10.7	2.4	140	286	95
	120 "	10.3	2.2	140	288	95
21	control	10.5	2.6	142	290	136
	30 minutes	9.2	2.4	144	293	140
	60 "	26.2	2.1	140	288	128
	120 "	32.0	2.0	142	292	120

Table 6: Effects of carotid artery infusions of magnesium chloride on plasma levels of ADH, calcium, sodium, osmolality, magnesium, and mean arterial pressure.

Dog	Period	Plasma ADH uU/ml	Plasma Calcium mM/L	Plasma Sodium mM/L	Plasma Osmolality mOsmol/$K_g H_2O$	Mean Arterial Pressure mm H_g	Plasma Magnesium mM/L
22	control	21.4	2.8	142	290	108	1.3
	low Mg	17.7	2.8	138	284	106	1.7
	high Mg	18.5	2.8	142	288	100	2.0
	control	19.0	2.8	142	291	106	1.4
23	control	6.3	2.6	138	282	96	1.2
	low Mg	8.5	2.6	140	286	100	1.5
	high Mg	10.0	2.6	140	285	100	1.9
	control	7.4	2.6	142	289	90	1.2
24	control	4.7	2.4	146	296	98	1.3
	low Mg	4.7	2.4	142	290	102	1.6
	high Mg	3.8	2.4	138	285	98	2.1
	control	6.7	2.4	138	283	92	1.5
25	control	2.0	2.6	142	292	110	1.1
	low Mg	1.8	2.6	142	292	108	1.5
	high Mg	2.4	2.5	140	288	108	1.9
	control	2.0	2.6	142	293	106	1.2
26	control	11.4	2.8	148	299	110	1.2
	low Mg	10.6	2.7	142	290	106	1.6
	high Mg	11.2	2.8	144	295	106	2.0
	control	8.5	2.8	140	288	112	1.4

DISCUSSION

These experiments were undertaken to substantiate the theory originally suggested by Douglas and Poisner (1964) that calcium is a link in the release of ADH from the posterior pituitary. Since the theory was developed from in vitro evidence, I wanted to see if calcium could cause the release of ADH from an intact animal.

The preliminary experiments shown in Table 1 demonstrate two things. First that the infusion of $CaCl_2$ can cause an increase in the plasma ADH level of anesthetized dogs. Second that it was not possible to compare carotid artery and femoral vein infusions of calcium in the same animal since the control values of ADH did not remain stable over long periods of time in these preparations. The rise in control values of ADH seen in dogs 3, 5, and 6 could be explained by the changes in respiratory function. None of the dogs used in the preliminary experiments were breathed on a respiratory pump. It is conceivable that changes in gas concentration due to hypoventilation could have directly caused the increase in ADH. It should also be mentioned that the decrease in pH that occurs during hypoventilation causes an increase in the ratio of free to bound calcium. If it is the concentration of free calcium that causes the release of ADH this could explain the increase in plasma ADH seen toward the end of these experiments. It is also possible that the increase in ADH is due to some change in cardiovascular function. For instance a decrease in left atrial pressure (Grauer and Henry, 1963) can cause an increase in ADH even though there is no change in mean arterial pressure. In dog 6 the fall in mean arterial pressure could explain the increase in plasma ADH level.

Since it was not possible to compare carotid artery and femoral vein infusion of $CaCl_2$ in the same dog, the comparison was made in different dogs. Figure 3 clearly shows that the arterial infusion of $CaCl_2$ caused an increase in plasma ADH. It also shows that calcium was more effective in increasing the level of ADH when infused in the

carotid artery than when the same dose was infused in the femoral vein. The level of plasma ADH after the carotid infusion of the lower dose of $CaCl_2$ is statistically different from control at the p= .02 level. The level of plasma ADH after the same dose was infused in the femoral vein is not significantly different from control (p= .6). Tables 2 and 3 show that the increase in plasma ADH was not a secondary result of increased plasma sodium, plasma osmolality, or decreased mean arterial pressure. Since both infusions caused the same increase in plasma $CaCl_2$ it is reasonable to conclude that the site in the body which is sensitive to changes in $CaCl_2$ concentration is located in the head above the level of the common carotid arteries.

The results from the ventriculo-cisternal brain perfusions, shown in Figure 4, are compatible with the hypothesis that the posterior pituitary is the area of the brain which is sensitive to the changes in $CaCl_2$ concentration. In the resting state the concentration of $CaCl_2$ in the extracellular fluid of the brain is the same as the concentration of $CaCl_2$ in the CSF. Extra calcium added to the CSF will diffuse through the extracellular fluid of the brain. The closer an area of the brain is to the ventricular system, the sooner it will show an increase in the concentration of calcium in its extracellular fluid. The perfusion of the ventricular system with an artificial cerebrospinal fluid which had an increased calcium concentration produced no increase in plasma ADH. This would indicate that the site of the brain sensitive to changes in calcium concentration is not immediately adjacent to the ventricles. The percentage increase in the calcium concentration of the CSF was the same as that observed in the plasma after carotid and venous infusions of calcium. The control value of CSF calcium concentration, however, is only half the concentration in plasma. Even though the percentage change in calcium was the same during ventriculo-cisternal perfusion and carotid infusion of calcium, the absolute value of the calcium concentration is different. Thus, it is possible that the ventriculo-cisternal perfusion of calcium did not elicit a rise in ADH

in the same manner as the carotid artery infusion due to the difference in the calcium concentrations that were used. However, since there is a blood brain barrier to calcium, one would not expect the higher concentration of calcium in the plasma to cause a correspondingly higher concentration in the extracellular fluid of the brain, even in the areas immediately adjacent to the capillaries (Graziani, et al., 1967). Only through direct measurement of the concentration of calcium in the extracellular fluid of the brain during brain perfusions and carotid artery infusions would one know the concentrations achieved.

Figure 4 shows that a 25 mM/L increase in the NaCl concentration of the solution perfusing the brain caused a marked increase in the level of plasma ADH. This could mean that the increase in osmolality was a more potent stimulus than the increase in calcium. It could also mean that the receptor site for changes in osmolality is different from the site responsive to changes in calcium. The finding by Douglas and Poisner (1964) that hypertonic saline does not elicit the release of ADH from isolated neurohypophysis whereas calcium is a potent stimulus in the same system would support the second interpretation.

The posterior pituitary is not readily accessible to short term changes in CSF concentrations. My findings are compatible with the hypothesis that $CaCl_2$ acts at the nerve terminals in the posterior pituitary and that hypertonic saline acts at a site more accessible to changes in CSF concentration, possibly an area in the hypothalamus.

Thyrocalcitonin was given to four dogs in an attempt to lower plasma calcium. As can be seen from Table 5 the calcium concentration of plasma was significantly lowered. This, however, did not cause a lowering of plasma ADH levels as expected. In fact, the drop in plasma calcium was concomitant with an increase in plasma ADH. It is possible that the increased level of circulating ADH was not a direct result of the lowered calcium levels, but occurred secondarily as a response to altered cardiovascular function. Following the injection of thyrocalcitonin, mean arterial pressure was reduced in all but one dog.

Since it has been shown that reductions in mean arterial pressure lead to increased levels of ADH (Rocha, Silva, and Rosenberg, 1969), this could explain the increase in ADH following the administration of thyrocalcitonin. Another possibility is that thyrocalcitonin directly caused the release of ADH. However, this seems unlikely since the peak rise in ADH was seen two hours after the intravenous injection of thyrocalcitonin. It should be mentioned that there are almost no studies in the literature showing a drop in plasma ADH levels from the normal control values, with the exception of water diuresis. Thus, it is possible that a certain basal level of ADH secretion is maintained via some type of regulatory system sensitive to plasma ADH levels. If this is the case, it would be very hard to show a fall in plasma ADH.

Douglas and Poisner (1964) found that if they increased the magnesium concentration in the incubation medium of isolated neurohypophysis they could inhibit the release of ADH. However, they needed to increase the magnesium concentration ten to twenty fold to observe the phenomenon. Since magnesium is a potent vasodilator, I could not raise plasma magnesium by such large amounts. At a lower dose of magnesium I was not able to produce any change in plasma ADH. Lesnik, Guzek, and Truczyk (1969) found that the infusion of $MgCl_2$ caused a decrease in the antidiuretic activity of material collected from the exposed pituitary stalk of rats. The difference between my data and theirs could be due to the fact that I was using intact animals and they were not. It is also possible that magnesium inhibits ADH secretion in the rat but not in the dog.

My experiments show that calcium may be involved in the regulation of plasma ADH levels in the anesthetized dog. This is not to imply that plasma levels of ADH are regulated by variations in the calcium concentration of the plasma. The calcium concentration of plasma is normally kept exceedingly constant. Rather the data are compatible with the hypothesis that calcium is involved in the mechanism by which ADH is released from the posterior pituitary gland. In

combination with the in vitro findings of Douglas and Poisner (1964), and the in vivo evidence of Thorn, Smith, and Skadhauge (1965), Dyball (1968), and Lesnik, Guzek, and Truczyk (1969), my findings lend support to the hypothesis that the entry of calcium into the posterior pituitary cells during depolarization is a link in the releasing mechanism for ADH.

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