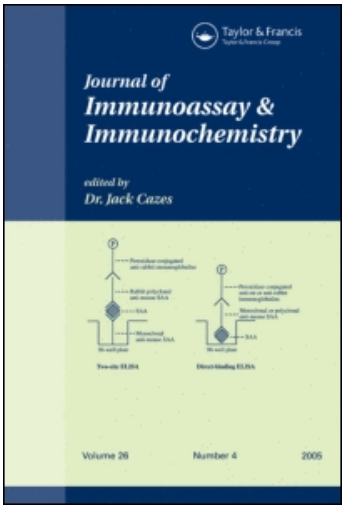

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A CYTOCHEMICAL BIOASSAY FOR ARGININE VASOPRESSIN : PRELIMINARY STUDIES

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ABSTRACT

A new method of measuring vasopressin activity is described. It depends on the finding that the Na^+-K^+-ATPase activity, measured cytochemically, in the thick ascending limb of the loop of Henle in rat renal tissue maintained in vitro, responded to increasing concentrations of synthetic arginine vasopressin in a log-dose related fashion. The limit of sensitivity was 0.002 pg/ml (2×10^{-15} mol/l). The dose-responses were reproducible; the inter-assay coefficient of variation was 6.4% at a vasopressin concentration of 0.02 pg/ml. Normal plasma stimulated this Na^+-K^+-ATPase activity, the stimulation being reduced by 98% when the plasma had been treated with an antiserum specific for vasopressin. Measured in this system, the circulating levels of plasma vasopressin, in healthy adults after 18h dehydration, was 4.0 ± 0.3 pg/ml (mean $\pm$ SEM; n=4) and fell to 0.6 ± 0.1 pg/ml following a water load. Absolute plasma vasopressin values obtained by the cytochemical bioassay were comparable to those measured by radioimmunoassay ($r = +0.97$, $p < 0.001$).

INTRODUCTION

Measurement of arginine vasopressin, namely the human antidiuretic hormone, has remained technically difficult particularly when applied to plasma. This is due in part to the extremely low circulating concentrations of the hormone, of the order of 10^{-12} mol/l, and in part to non-specific interference in immunoassays by substances of large molecular weight.

Classical bioassays, based on changes in diuresis in water-loaded, ethanol anaesthetized rats (1) are subject to considerable inter-assay variation and interference from vasopressin-independent mechanisms altering diuresis; radioimmunoassays require extraction of blood samples before assay and necessitate antisera of high avidity (2). Because of the low circulating levels, both bio-and immunoassays tend to be operated at the limits of their sensitivity. Despite these methodological difficulties, considerable advance in our understanding of the physiology and pathophysiology of vasopressin secretion has been achieved, but vasopressin assays tend to remain research tools. There clearly is a place for a precise method, free from non-specific interference, and yet sufficiently sensitive to detect low circulating levels of vasopressin.

The technique of cytochemical bioassay has been available for a few years (for reviews see ref. 3,4) and has been applied to the measurement of many peptide hormones (4). The method combines biological specificity for the hormone peptide with remarkable sensitivity, and would appear to be suitable for overcoming some of the problems associated with the measurement of vasopressin in body fluids. We describe below our preliminary observations in developing a cytochemical bioassay for arginine vasopressin and its application to the measurement of this hormone in plasma.

METHOD

Female Wistar rats (200-300g) were allowed free access to food and water before they were killed by nitrogen asphyxiation. Kidneys were quickly removed and the renal capsule was stripped carefully from each kidney which was then bisected longitudinally. Three segments were cut from each half of the kidney. Each segment was placed, cortical surface downwards, on a de-fatted lens tissue which lay on a metal grid table in a vitreosil dish containing Trowell's T8 culture medium, pH 7.6, up to the level of the lens tissue (5). The dish was placed in a culture pot;

the air within the pot was displaced by 95% oxygen and 5% carbon dioxide, after which the pot was sealed. Kidney segments were maintained in culture at 37°C for 5h (3).

After this period the vitreosil dishes were removed from the culture pots and the culture medium was discarded. Solutions of synthetic arginine vasopressin (Ferring AB Pharmaceuticals), or plasma diluted suitably with Trowell's T8 medium were poured over the segments. Preliminary experiments defined the optimal time required for the maximum stimulation of enzyme activity, and thereafter all vasopressin and plasma solutions were allowed to react for either 5 or 17.5 min. After application of either the standard preparation of hormone or diluted plasma, each segment was chilled to -70°C in n-hexane ("free from aromatic hydrocarbons" grade, boiling range 67-70°C) for 30 sec, and was then quickly transferred, by means of cold forceps, into glass tubes pre-cooled to -70°C. Although the enzyme activity of the tissue remained stable for at least 48h, segments were sectioned within 24h. Each segment was mounted on a microtome chuck so that each section contained renal pelvis, medulla and cortex. Sections were cut at 10 μm in a low temperature cryostat (Bright, Model FS/FAS/M/LT). Constant thickness of sections was ensured by an automatic cutting device that controlled the speed at which sections were cut (4,6). The handle of the knife was cooled to -70°C to facilitate the transfer of sections from the knife on to glass slides (7).

The enzyme activity was measured immediately after the segments were sectioned. The method for assaying magnesium-activated Na^+-K^+-ATPase activity was based on the method of Schwartz *et al* (8) except that the concentration of all chemical constituents were increased four-fold to overcome the poor diffusion of substrates within the polypeptide medium. The final method used to measure Na^+-K^+-ATPase activity, its optimization and validation, are described in detail elsewhere (9). In brief, the

reaction mixture for the measurement of total ATPase activity was 40% solution (W/V) of polypep 5115 (Sigma) in 200 mmol/l Tris buffer at pH 7.4 containing sodium chloride (24 mg/ml; 40 mmol/l), magnesium chloride (4 mg/ml; 20 mmol/l), potassium chloride (2.8 mg/ml; 37.5 mmol/l), disodium adenosine 5' triphosphate (10 mg/ml; 16.5 mmol/l; Boehringer), lead ammonium citrate/acetate complex (32 mg/ml; Sigma), and sodium acetate (1 mmol/l). For measurement of ouabain-insensitive enzyme activity, ouabain octahydrate (0.3 mg/ml; 0.4 mmol/l; Sigma) was added to the reaction medium. The final pH of both solutions was adjusted to pH 7.65 by the addition of sodium and potassium hydroxides (10:1 on a molar basis; 2.5 mmol/l) or of 0.1 mmol/l hydrochloric acid. Morphology of the tissue in some sections was assessed after staining with toluidine blue.

Enzyme activities were measured at 37°C. The reaction mixture was poured into small perspex rings which had been placed over each section. Ouabain-insensitive ATPase activity was measured on alternate serial sections. After 15 min, the rings were removed and the sections were rinsed several times in fresh 200 mmol/l Tris buffer, pH 7.4 at 37°C to remove all traces of the reaction mixture. The sections were then immersed, at room temperature, in distilled water that had been saturated with hydrogen sulphide for 2 min. They were rinsed several times in distilled water and allowed to dry at room temperature before mounting in Farrants' medium.

The reaction product was measured by means of a scanning and integrating microdensitometer (Vickers, Model M85) at 550 nm, with a (x40) objective and a flying spot of 0.5 μm diameter. The size of the mask was sufficient to encompass one cell. Twenty cells from different thick ascending limbs of Henle in the medullary region of the kidney were measured in duplicate sections. After calibration of the instrument (4), readings were expressed as the mean±SEM of the integrated extinction. Total ATPase and ouabain-in-

sensitive Na^+-K^+-ATPase activities were recorded. The value for ouabain-sensitive Na^+-K^+-ATPase activity was derived by subtraction of these values.

Studies were designed to detect the time of the peak activities of total and ouabain-sensitive ATPase in response to synthetic arginine vasopressin diluted in Trowell's T8 medium to 0.02 pg/ml. This was achieved by allowing vasopressin solutions to react with segments for various time intervals. Thus, in the initial studies, segments were chilled after they had been exposed to vasopressin for 0.5 to 10 min at 1 min intervals; this study was repeated using 2.5 min intervals up to 20 min. After defining the time of the peak enzyme activities at this concentration, the effects of varying the concentration of vasopressin within the range 0.002 to 2.0 pg/ml, upon the timing of the peak response, was assessed. Total and ouabain-sensitive ATPase activities were measured in response to solutions of vasopressin (0.002 to 2.0 pg/ml) at the 2 times of maximum activity. Subsequent studies examined the effect of higher and lower concentrations of vasopressin on the enzyme activities at the times of maximum response.

Venous blood from a healthy adult, dehydrated for 18h, was taken into heparinized tubes pre-cooled to 4°C. The blood was immediately centrifuged for 10 min at 2000 rpm in the cold. The plasma was separated and divided into two 1 ml aliquots. Antiserum specific to vasopressin, the characteristics of which have been well defined (10), was added to one aliquot at a final dilution of 1:6000. Both aliquots were stored on ice for 10 min before being diluted 1:20 and 1:2000 with Trowell's T8 medium, after which they were applied to the kidney segments, maintained *in vitro* as performed with the standard preparation of vasopressin. Finally, plasma was obtained from four healthy individuals after 18h dehydration and subsequent oral water loading with 20 ml/kg to assess the effect of dehydration and water loading on the biological action of plasma on ouabain-sensitive ATPase.

RESULTS

The optimal pH of the vasopressin solutions to stimulate maximum ATPase activity in kidney segments was pH 7.65. The time course of the response of the cells in the thick ascending limbs, in segments, to synthetic arginine vasopressin at 0.02 pg/ml showed maximal total ATPase and ouabain-sensitive (Na^+-K^+-ATPase) ATPase activities after 5 and 17.5 min exposure to the hormone (Fig. 1). There was no significant difference in the time of the second peak of enzyme activity in response to variations in vasopressin concentration from 0.002 to 2.0 pg/ml. Total and Na^+-K^+-ATPase

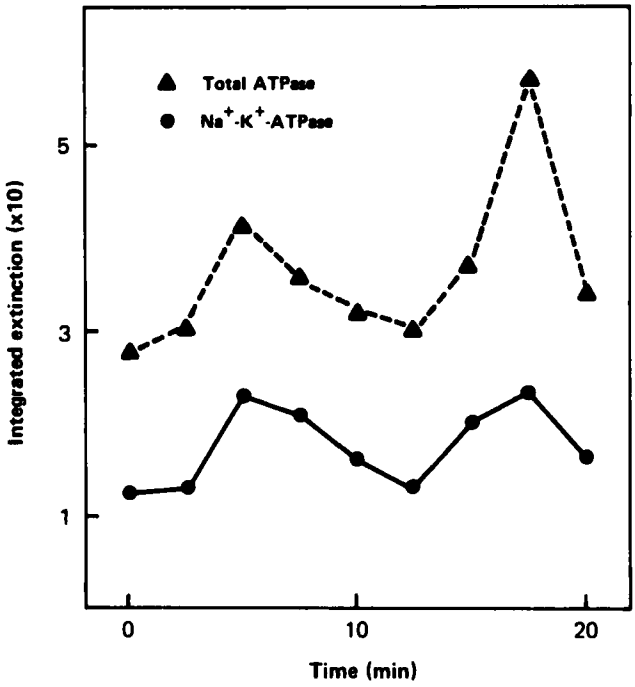

FIGURE 1. Time course of total and Na^+-K^+-ATPase responses in rat renal segments exposed to synthetic arginine vasopressin (0.02 pg/ml). The mean ATPase activities are expressed as the mean integrated extinction recorded in 20 renal tubular cells of the ascending limb of Henle from duplicate sections at each time interval.

activities did not decrease further than the value obtained with a vasopressin concentration of 0.002 pg/ml despite lowering the concentration to 2×10^{-6} pg/ml. Increase in vasopressin concentration above 2.0 pg/ml caused a fall in both ATPase activities. Addition of Trowell's T8 medium alone to segments did not stimulate ATPase activities.

Arginine vasopressin gave a linear log-dose response over the range 0.002 to 2.0 pg/ml after exposure to the hormone for 17.5 min (Fig. 2). The slopes of the lines for total ATPase and Na^+-K^+-ATPase were not significantly different (0.048 and 0.046 respectively, $p > 0.05$). The correlation coefficient for the points on the Na^+-K^+-ATPase line was 0.99; for the total ATPase is was 0.97. The interassay coefficient of

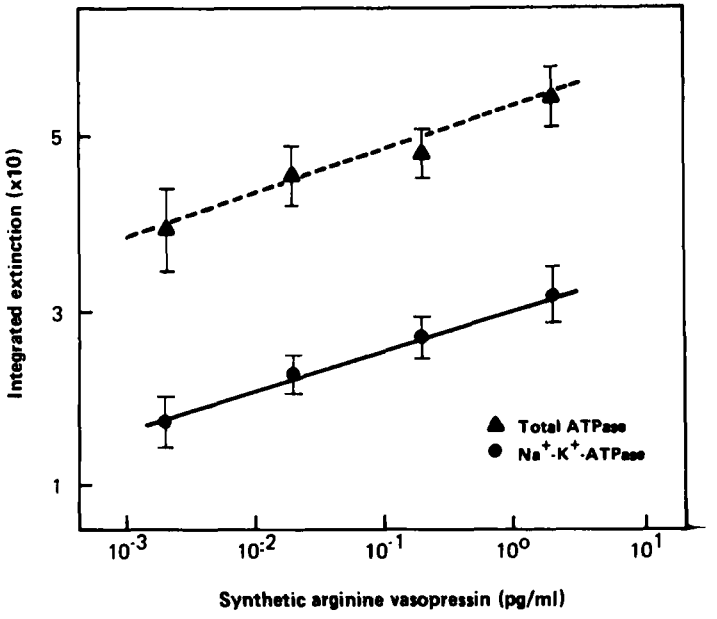

FIGURE 2. The dose-response graph of total and Na^+-K^+-ATPase activities in rat renal tubular cells of the ascending limb of Henle induced by increasing concentrations of synthetic arginine vasopressin, expressed as mean ± SD of 5 experiments.

variation of the individual concentrations of vasopressin ranged from 6.3 to 14.4% for Na^+-K^+-ATPase and 4.7 to 16.9% for total ATPase activities.

Addition of normal plasma at 1:20 and 1:2000 dilutions to segments gave Na^+-K^+-ATPase responses that were parallel to the standard curve (Fig. 3). Antibody specific to synthetic arginine vasopressin, when added to plasma, reduced the plasma vasopressin concentration from 6.7 to 0.05 pg/ml. The enzyme activity remaining in plasma after the addition of specific antiserum diluted parallel to the standard curve.

Results of plasma vasopressin concentration after 18h dehydration and subsequent water loading are given in Fig. 4. Plasma vasopressin fell from 4.0±0.3 (mean ± S.E.) to 0.6±0.1 pg/ml after the water load.

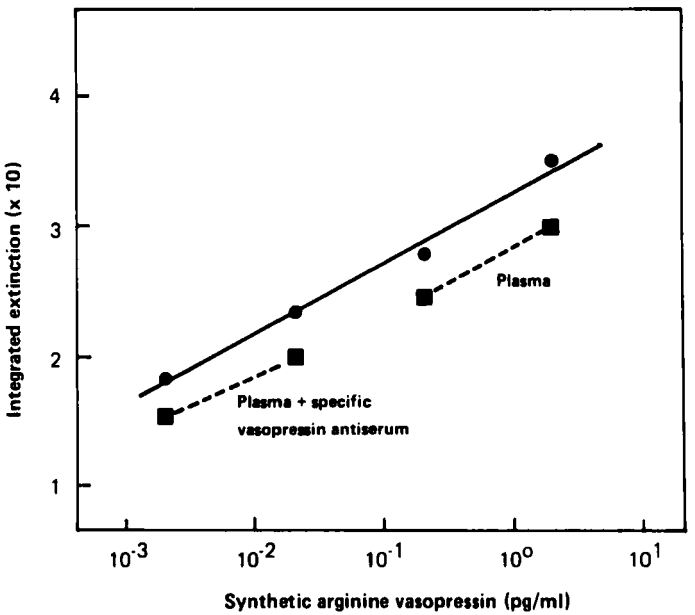

FIGURE 3. Na^+-K^+-ATPase responses to human plasma (filled squares) at 1:20 and 1:200 gave values parallel to the standard dose-response graph (filled squares). Addition of specific vasopressin antiserum to plasma (open squares) reduced enzyme activity.

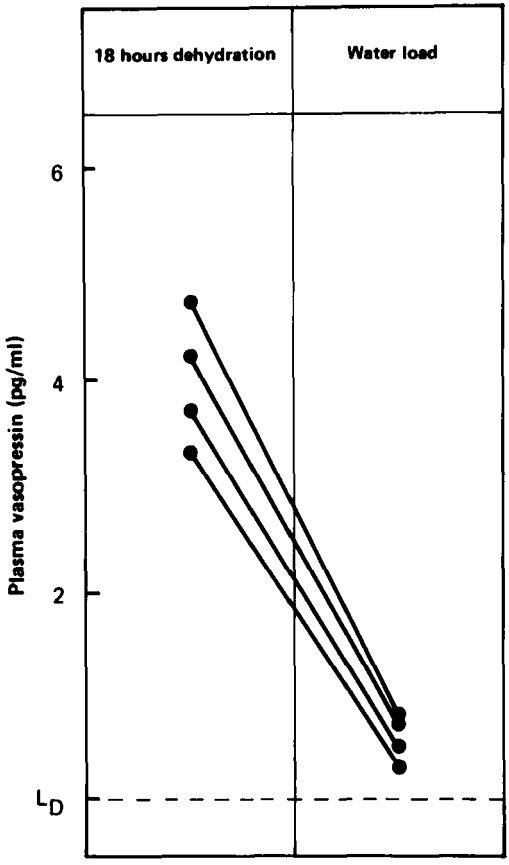

FIGURE 4. Results of plasma vasopressin measured by changes in Na^+-K^+-ATPase activities in rat renal tubular cells, obtained from 4 healthy volunteers after 18 h dehydration and followed by a standard oral water load.

Immunoreactive plasma vasopressin concentrations in the same samples, measured by a well-established radioimmunoassay (10), were 4.5±0.5 and 1.7±0.2 pg/ml after dehydration and water load respectively. In individual samples, there was a highly significant positive correlation between the plasma vasopressin concentration measured by cytochemical bioassay and the

concentration measured by radioimmunoassay (correlation coefficient $r = +0.97$, $t = 9.8$, $p < 0.001$).

DISCUSSION

Although ATPase appears to be widely distributed throughout the mammalian kidney, particularly high concentrations have been reported in the proximal convoluted tubule, the medullary thick ascending limb of Henle, and the distal convoluted tubule (11). Our previously reported observations agreed with these findings (9). For the development of a cytochemical bioassay of vasopressin we confined our observations to the medullary region of the kidney. Synthetic arginine vasopressin caused a distinct increase in both total and Na^+-K^+-ATPase activities located in the renal medulla, with peak responses occurring after 5 and 17.5 min exposure to the hormone. The precise physiological significance of two peaks remains unclear, but similar phenomena have been described with other peptide hormones (12). Reproducible standard curves over the range of concentration of vasopressin of 0.002 to 2.0 pg/ml have been obtained using principally the 17.5 min exposure time. This suggests that changes in Na^+-K^+-ATPase activity might provide a stable and specific assay to assess vasopressin concentration. However, the latter claim needs to be substantiated by further work investigating the effect of other hormones and substances known to act on the nephron.

Nevertheless, our results do indicate that there is a vasopressin-sensitive ATPase in the rat nephron. The renal collecting ducts are believed to be the target tissue for vasopressin but very little Na^+-K^+-ATPase activity has ever been observed in these structures (11). Our findings imply that vasopressin may also be acting at sites in the renal medulla other than the collecting duct. The recent work by Imbert-Teboul *et al* (13) tends to support our argument. These investigators

reported membrane-associated adenyl cyclase that was sensitive to vasopressin not only in the collecting duct but also in significant quantities in the thick ascending limb of Henle. Vasopressin-sensitive adenyl cyclase was not found elsewhere in the nephron. We would therefore contend that a vasopressin-sensitive Na^+-K^+-ATPase is located in the thick ascending limb, and, in fact, the morphology of the cells containing this ATPase confirms our contention (9). If our hypothesis is correct, then the physiological consequences are logical. For vasopressin to act as an antidiuretic hormone by increasing the permeability of collecting duct cells to water, it is essential that the renal medullary interstitial tissue is hypertonic to allow free water to pass down a concentration gradient (14). The pumping of Na^+ ions into the medullary interstitial tissue by an ATPase would increase its tonicity, and thereby enhance the antidiuretic effect.

Dilutions of fresh plasma applied to renal segments stimulated Na^+-K^+-ATPase activity, the results running parallel to the standard curve. Although many plasma substances could have been responsible for this enzyme stimulation, the observation that antiserum specific for vasopressin substantially reduced ATPase activity strongly suggests that, under the conditions we have described, the stimulation of Na^+-K^+-ATPase activity by plasma is probably due to vasopressin. Clearly further studies are required to confirm specificity for vasopressin since it is well-recognised that parathyroid hormone, thyroxine and probably many other plasma constituents, affect numerous enzymes at various sites in the nephron (15).

Plasma osmolality is the major determinant of vasopressin secretion (16). Dehydration and water loading are standard methods of stimulating and inhibiting secretion of vasopressin. The results of the cytochemical measurement of vasopressin-like activity were in agreement with those anticipated changes in the secretion of vasopressin, thus providing some

physiological validation for the assay. Of particular interest is the observation that the absolute concentrations of plasma vasopressin are very similar to values obtained by radioimmunoassays reported by many investigators (for review see ref. 17). This is in contrast to other hormones, for example parathyroid hormone, where hormone concentrations measured by cytochemical methods are considerably lower than values obtained by radioimmunoassay (18).

Our preliminary studies indicate that the rat nephron has a Na^+-K^+-ATPase, probably located in the thick ascending limb of Henle, that is sensitive to vasopressin. Results obtained from our physiological studies and from our observations on the effect of vasopressin antiserum suggest that the method is specific for vasopressin. The method is extremely sensitive and requires no extraction of plasma which is common to the majority of bioassays and radioimmunoassays for vasopressin in current use.

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