INHIBITORY ACTION OF CALCIUM ON THE INACTIVATION OF ANTIDIURETIC HORMONE BY RAT KIDNEY SLICES

By

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ABSTRACT

Increasing the calcium concentration 5 times or more in the medium used for studying the inactivation of arginine-vasopressin by rat kidney medulla slices caused a marked inhibition of the inactivating activity of such slices. This effect was not found in homogenates of rat kidney medulla. The results are in agreement with the interpretation that the high calcium concentration decreased the cellular permeability to the hormone. This would seem to give a rational explanation of the vasopressin-resistant diabetes insipidus which is found in hypercalcaemia.

Some evidence has been presented in favour of the hypothesis that calcium is involved in the mechanism of action of antidiuretic hormone (ADH) on the mammalian kidney (Thorn 1960, 1961). In a series of tests of this hypothesis, we have examined the effect of increasing the calcium concentration in the medium used for investigating the inactivation of arginine-vasopressin (ADH of the rat) by rat kidney slices.

METHODS AND MATERIAL

The method of Thorn & Willumsen (1963) was used for studying inactivation of antidiuretic hormone by rat kidney slices. One of the essential features of this method is the division of the slices into zones.
It has been shown that the rate of inactivation of both arginine- and lysine-vasopressin in slices from papillary tissue is about twice that found in cortical tissue (Thorn & Willumsen 1963). In "whole kidney" slices the relative proportions of the different zones may vary considerably. Hence, it seemed essential for the demonstration of experimentally induced changes in the rate of inactivation that slices from homogenous zones should be used. In the present study slices from the outer and the inner medulla (zone 2 and 3), respectively, were investigated.

Another essential feature of the method is the use of a medium with a composition very similar to that of rat extracellular fluid. In this connection it should particularly be emphasized that the calcium concentration of this medium is 2.5 mM. It is known that smaller concentrations of calcium than 2 mM do not prevent a loss of enzymes to the medium (Krebs et al. 1963).

The ordinary medium described previously (Thorn & Willumsen 1963) was modified in different ways. In one set of experiments, the calcium concentration of the medium was increased 5 times, the strength of the phosphate buffer at the same time being reduced to 1/5 the ordinary value. The osmolality of the medium was kept unchanged (approx. 290 mOsm/kg) by reducing the concentration of sodium chloride. In another set of experiments, the concentration of calcium in the medium was increased 10 times, the buffer strength and sodium chloride concentration being reduced as described previously. The ordinary medium of Robinson used for most of the studies is buffered with phosphate (8 mmol/l). In a few experiments this was replaced with Tris buffer (40 mmol/l, \(p_H 7.2\)). The experimental procedure was essentially as described previously (Thorn & Willumsen 1963). In brief, approximately 50 mg (wet weight) of slices from each zone was incubated with 200 mU (milliunits) of a commercial preparation of arginine-vasopressin (Pitressin, Parke, Davis & Co., lot no. T 102LA) in 2 ml medium.

The incubation was carried out at 37° C, aerobically in Warburg vessels for 2 hours (after an equilibration period of 30 min). For each zone one set of slices was incubated with the hormone in the ordinary medium, and a parallel set of slices was incubated with the medium containing a high concentration of calcium.

In 10 experiments the inactivation of arginine-vasopressin by homogenates from rat kidney medulla was studied. 10% homogenates of papilla + outer medulla were made in 0.25 M sucrose. 0.2 ml of homogenate was quickly added by a wide-mouthed pipette to each of 2 Warburg vessels containing 200 mU of arginine-vasopressin plus 2 ml of the ordinary medium or a medium having a calcium concentration 10 times higher. Incubation was carried out at 37° C aerobically for 1 h after which 0.2 ml of 3% acetic acid was added (Dicker & Greenbaum 1956). The tubes were heated on a boiling water bath for 3 minutes and centrifuged. The cool supernatants were used for assay.

**RESULTS**

The effects of increasing the calcium concentration in the medium 5 and 10 times on the rate of inactivation of arginine-vasopressin by the slices is seen in Table 1.
Effect of increasing the calcium concentration in the medium on the rate of inactivation of arginine-vasopressin by rat kidney slices.

(Medium buffered with phosphate).

<table>
<thead>
<tr>
<th>Increase in Ca conc. in medium</th>
<th>5 ×</th>
<th>10 ×</th>
</tr>
</thead>
<tbody>
<tr>
<td>zone 2</td>
<td>90</td>
<td>0</td>
</tr>
<tr>
<td>zone 3</td>
<td>93</td>
<td>85</td>
</tr>
<tr>
<td>zone 2</td>
<td>72</td>
<td>45</td>
</tr>
<tr>
<td>zone 3</td>
<td>110</td>
<td>70</td>
</tr>
<tr>
<td>zone 2</td>
<td>70</td>
<td>72</td>
</tr>
<tr>
<td>zone 3</td>
<td>75</td>
<td>45</td>
</tr>
<tr>
<td>zone 2</td>
<td>80</td>
<td>49</td>
</tr>
<tr>
<td>zone 3</td>
<td>80</td>
<td>75</td>
</tr>
</tbody>
</table>

The oxygen consumption of the slices was essentially the same in the different media. Both when a normal and when a 5 or 10 times increased concentration of calcium was used, there was in some experiments a decrease in $p_H$ of 0.1–0.2 units. These changes, however, were not constant and were not correlated with the inhibition of vasopressin inactivation. In 4 experiments with a calcium concentration 5 times normal in which Tris buffer (40 mmol/l) replaced the phosphate buffer, no change in $p_H$ occurred in any of the zones. In these experiments the amount of hormone inactivated in per cent of the control was 71 and 53 for zone 2 and 3, respectively. These results would seem to exclude the possibility that differences in the $p_H$ could explain the inhibition. In 2 experiments with an increase in the calcium concentration of the medium 10 times normal inhibition of the inactivation of lysine-vasopressin (Insipidin®) to activities of 50% of the control value were found in zone 2 and 3.

Inhibition of the swelling of the papillary slices seemed to parallel the inhibition of the inactivation. Normally, papillary slices incubated with arginine-vasopressin for 2 hours swell considerably. The wet/dry weight ratio of papillary slices incubated with the hormone is 10.1 whereas without hormone it is only 8.9. In the experiments with the calcium concentration increased 10 times, the average wet/dry weight ratio of the slices from zone 3 was 95% of the ratio found in the control slices. This would seem to indicate an inhibition of this probable in vitro effect of the hormone.

In 10 experiments the inactivation of arginine-vasopressin by homogenates from rat kidney papillae was studied. The rate of inactivation of arginine-vasopressin by a homogenate having a calcium concentration 10 times normal was compared with the rate found in a homogenate having a normal calcium concentration. No difference was found between the 2 sets of homogenates in 9 of the 10 experiments.
DISCUSSION

For a very long time it has been known that hypercalcaemia caused by hyperparathyroidism, neoplasms, overdose of vitamin D or injection of calcium causes severe inhibition of the renal concentrating mechanism in mammals (a vasopressin-resistant diabetes insipidus). Obvious explanations for this effect would be that calcium interferes with the mechanism of action of antidiuretic hormone either directly by inhibiting its action at the receptors or indirectly by inhibiting the access of the hormone to the receptors by causing a decrease in the permeability of the cells to the hormone.

Another possible explanation of the defect in the concentrating mechanism would be that the normal gradient for sodium in the kidney medulla is disturbed by hypercalcaemia. Manitius et al. (1960) demonstrated that in rats with experimental hypercalcaemia there was some decrease in the sodium concentration of the papilla. The fall in the maximum urine osmolality was, however, greater than the fall in the sodium concentration in the papilla, a fact which might indicate that in these experiments there was also some decrease in the diffusion of water across the distal tubular part of the nephron. Recent studies by Zeffren & Heinemann (1962) have shown that the effect of calcium on the concentrating ability is reversible and probably not due to any abolition of the renal medullary sodium gradient, but to an interference with the function of the cell membranes in the distal tubules.

Of importance in the discussion of the possibility that hypercalcaemia interferes directly with cell permeability to the hormone are the reports of Bentley (1959), Whitembury et al. (1960), Petersen & Edelman (1962), Pigeon & Epstein (1963), Herrera & Curran (1963) and Curran et al. (1963) that calcium may interfere with the permeability to water, urea and sodium in isolated toad bladders or intact frog skin under circumstances in which no change has taken place in the osmotic gradient. These findings together with the well-known effects of calcium on water permeability exerted directly on cells would suggest that hypercalcaemia, at least in part, produces its effect on the concentrating mechanism in mammals by interfering with the permeability of the distal tubular cells.

It would appear from the results of the present experiments that the inactivation of antidiuretic hormone is inhibited by elevation of the calcium concentration in the medium. Results of a similar nature have been found in a set of experiments carried out independently by Dr. M. Smith, Cambridge (Smith 1963). Smith used a method in which the reference factor was the wet weight of the slices before incubation. We used the »final dry weight« of the slices as a reference factor. For several reasons this would seem to be a better reference (Thorn & Willumsen 1963). One might consider whether calcium concentrations lower than 2.5 mm should be tried in order to see, whether under such circumstances, a stimulation of the inactivation (in comparison with
our base-line value) could be found. Such low concentrations were used by Smith. This, however, was not considered satisfactory since at lower calcium concentrations than 2 mM intracellular enzymes leave the slices (Krebs et al. 1963) when incubation is carried out at 37°C as in our method. As previously discussed (Thorn & Willumsen 1963) this leads to inaccuracies in the determination of the rate of inactivation.

It is apparent from the results listed in Table 1 that there seemed to be no significant increase in the inhibition when the concentration of calcium was raised from 5 to 10 times that in the control medium (2.5 mM). This is in agreement with the fact that Smith (1963) found maximum inhibition of the inactivation (a decrease to approximately 50% of the control value) with a concentration of calcium of 7.5 mM (3 times our control value). Increase of the calcium concentration to 15 mM had no further effect in his experiments.

The considerable variation found in the inhibition with concentrations of calcium both 5 and 10 times normal probably reflects either individual differences or differences in the opportunity for calcium to reach the cells, determined by the different architecture of the slices.

It might be argued that a study of the inactivation of vasopressin should be done by determining the apparent first-order rate constant for the inactivation of the hormone and that investigations of the possible inhibitory effect of various substances should be done, determining Lineweaver & Burk (1934) or Dixon (1953) plots for deciding whether or not any inhibition might be competitive. Such procedures were – for various reasons – not considered to be necessary in the present study. It would appear that there are at least two enzymes responsible for the inactivation of vasopressin in kidney slices, one an SH enzyme which attacks the SS link and another which attacks the amide groups in the molecule (Dicker 1961). Neither of these enzymes have been purified, but their existence has been suggested by studies on fractionations of kidney homogenate. Thus the intimate nature of the enzymic inactivation has still to be explored. Furthermore, the inactivation of the hormone in a method using kidney slices is a complicated one. Besides the kinetics for the two enzyme systems involved complicated relations are due to the employment of a semi-intact tissue where the hormone has to enter by routes presumably very different from those taken in the intact organism. In the present system the hormone would seem to have to pass some diffusion barriers in the form of the cell membranes. This together with the fact that some enzyme probably leaks into the medium, further complicates the method. Finally, determinations of the velocity constants would require the use of very large hormone concentrations which would change during the period of investigation when samples are withdrawn. For these reasons it was decided to use a very simple procedure for expressing the inactivation in the slices and the inhibition caused by calcium.
The fact that high concentrations of calcium do not inhibit the inactivation of vasopressin by kidney homogenates is in accordance with the interpretation that the effect of calcium is due to a tightening of the membrane to the uptake of hormone.

The present experiments support the hypothesis that the hormone has to enter the cells of the kidney to become inactivated. This does not mean that it has to enter the cells to exert its action, but a number of facts seem to indicate that action and inactivation are intimately associated (Thorn & Willumsen 1963). Supporting this concept are the experiments of Leuschner (1960) who injected oxypressin (leucyl8-Vasopressin) into isolated guinea pig kidneys and afterwards separated the cell constituents by differential centrifugation. By this procedure the hormone was recovered mainly from the supernatant-microsome fraction. Objection could be taken against these findings on the ground that hormone might have come in contact with these particles after the rupture of the cellular walls. Several other findings of Leuschner, however, indicated that this was not the case.

No definite proof of entrance of ADH into the cells in other systems than the mammalian kidney has been obtained as yet, but it has been found by several investigators (Koefoed Johnsen & Ussing 1953; Hays & Leaf 1962) that ADH has to be added to the serosal side of the frog skin or toad bladder in order to produce an action, which is, however, exerted on the mucosal side.

Petersen & Edelman (1962) recently showed that the inhibitory effect of high concentrations of calcium on the action of ADH on the isolated toad bladder could be overcome by increasing the concentration of vasopressin in the medium. This fact is in accordance with the interpretation of our findings in kidney slices.

Neither the present experiments, nor those of Petersen & Edelman, however, exclude the possibility that calcium is a factor directly involved in the mechanism of action of ADH at the receptor level. Besides the experiments of Thorn (1960, 1961) which have been confirmed by Dicker & Eggleton (1961) and by Nielsen (1963), there is some evidence that calcium is necessary for the action of ADH in the frog skin (Herrera & Curran 1963). The effect of high concentrations of calcium found in the present experiments most likely take place at a stage preceding binding of hormone to the receptors.

It would appear from the results of the present experiments and those of Smith (1963) that hypercalcaemia inhibits the access of the hormone to the kidney cells. The rate of inactivation by kidney slices was reduced to between 1/2 and 2/3 the normal value. This might very easily produce a marked inhibition of the action of the hormone by a considerable derangement of the normal elimination pattern. Most vasopressin present in the blood entering the kidneys is normally cleared. Usually approximately 1/8 of an injected dose is inactivated in the kidneys and 10–15 1/8 excreted in the urine whereas the rest
is inactivated in the liver. If the binding of hormone in the kidney is inhibited, hormone left over in the plasma leaving the kidney will have the opportunity of being excreted in the urine at the next passage of the glomeruli or in the liver (although entrance to the liver cells might also be inhibited by hypercalcemia). Some facts seem to demonstrate that vasopressin acts on kidney tubule cells from the blood side (Skadhauge 1963) and that it is not secreted (Towbin & Ferrell 1963). Therefore, increased filtration of hormone would not be associated with increased antidiuretic effect.

The main implication of the results of the present experiments seem to be that, together with other findings, they strongly indicate the possibility that antidiuretic hormone has to enter kidney cells in order to have an effect on them and to become inactivated there. The vasopressin-resistant diabetes insipidus found in hypercalcemia can consequently most naturally be explained as due – at least in part – to a decreased ability of the hormone to enter the cells because of an unspecific tightening of the cell membranes by the high concentrations of calcium.

REFERENCES

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