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Effect of cAMP and Calmodulin Inhibitors on Water Absorption in Rat Proximal Tubule (41878)

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Abstract. The possible role of calmodulin in solute transport was examined in the kidney of the rat. Utilizing a radioimmunoassay, calmodulin was identified and quantitated in homogenates of the cortex of the kidney. The physiologic significance of these findings was examined utilizing *in vivo* microperfusion techniques applied to the proximal convoluted tubule of the thyroparathyroidectomized rat. The addition of dibutyryl cyclic adenosine monophosphate (cAMP) to the luminal perfusion solution resulted in a lower rate of water absorption of 1.67 ± 0.09 nl min^{-1} mm^{-1} as compared to 2.46 ± 0.11 in controls. The addition of either of two compounds with affinity for calmodulin, trifluoperazine (TFP) or W-13, reversed the cAMP-induced inhibition of water absorption. In the absence of cAMP, neither agent affected water absorption. Analogs of TFP and W-13 with lower binding affinities for calmodulin had no effect on water absorption and did not reverse the cAMP effect. None of the above experimental maneuvers affected the absorption of phosphate. These results demonstrate the presence of calmodulin in the kidney of the rat and suggest that calmodulin may be involved in cAMP-associated inhibition of water and electrolyte transport in the proximal tubule of the rat.

Calmodulin has been identified as a major calcium binding protein and has been implicated as a modulator of an impressive number of intracellular events. The protein itself has been identified in many species of animals and in many organs. Several recent reviews have cataloged the species and organs in which calmodulin has been identified (1, 2). These reviews also list the physiologic processes for which a role for calmodulin has been suggested (1, 2). Prior reports have documented the presence of calmodulin in renal tissue (3–5). Although a role for calmodulin in solute transport has been suggested in nonrenal tissue, we are unaware of reports implicating a role for this protein in the transport of electrolytes and water in the mammalian kidney (6, 7). The present studies provide evidence that calmodulin is present in the kidney of the rat. *In vivo* microperfusion studies indicate that the inhibition of water absorption by cyclic adenosine monophosphate (cAMP) in the proximal tubule of the rat is blocked by drugs which have affinity for calmodulin in other tissue systems. The results, then, implicate a role for a calmodulin-dependent step in electrolyte and water transport in the mammalian kidney.

Methods. Studies were performed on male Sprague–Dawley rats with free access to food and water prior to study.

Calmodulin determinations. The calmodulin concentration of homogenates of kidney cortex and brush border membranes was determined by radioimmunoassay (8). The preparation of the homogenates of kidney cortex and of brush border membranes by a modification of a magnesium aggregation method have previously been described (9). The methodology and characterizations of these membranes have also been reported from our laboratories (10). In brief, the brush border membranes as compared to the whole homogenate were enriched in alkaline phosphatase 10- to 13-fold but not enriched in the basolateral membrane marker Na^+–K^+ ATPase (10).

Micropuncture studies. Animals were anesthetized with pentobarbital sodium (50 mg/kg body wt) injected intraperitoneally. A thyroparathyroidectomy was performed utilizing electric cautery following which the animals were prepared for micropuncture. Two hours were permitted to elapse after thyroparathyroidectomy before the experiments were started. In all animals, the measured fractional

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excretion of phosphate was less than 2%, confirming adequate removal of all parathyroid tissue. Each animal received a volume of isotonic saline equal to 1% of body weight to replace surgical losses of fluid and a sustaining intravenous infusion of saline at a rate of 1.2 ml/hr for the duration of the study.

In vivo microperfusion studies were performed in the proximal convoluted tubule by methods previously described (11). The microperfusion solution (pH 7.4) contained sodium, 139 mmole/liter; chloride, 119 mmole/liter; bicarbonate, 25 mmole/liter; potassium, 4 mmole/liter; and calcium 0.5, mmole/liter. NaH_2PO_4 was added to the solution to a final concentration of 2 m*M*. [*methoxy*-^{3}H]Inulin and [^{32}P]orthophosphate (New England Nuclear Corp., Boston, Mass.) were added in amounts sufficient to permit accurate isotope counting. Where examined, dibutyryl cAMP (10^{-6} *M*), (Sigma, St. Louis, Mo.) and/or trifluoperazine (TFP) (30 μM), TFP oxide (30 μM), *N*-(4-aminobutyl)-5-chloro-2-naphthalenesulfonamide (W-13, 30 μM; CAABCO, Inc., Houston, Tex.) or *N*-(4-aminobutyl)-2-naphthalenesulfonamide W-12, 30 μM; (CAABCO) were added to the microperfusion solution. At the conclusion of each microperfusion, the tubule was filled with latex and the distance between perfusion and collection sites determined subsequently by microdissection. Generally, only one solution was examined in any individual animal and two to four microperfusion collections were obtained per animal. The procedures for processing of the microperfusion samples have previously been described (11). For each microperfusion, the perfusion rate, the rate of water absorption, and the rate of phosphate absorption were determined from standard formulas. All values are expressed as the means ± SEM for all tubules studied. The results were not different when calculated as the mean of means from individual animals. Statistical significance was determined by the *t* test for unpaired data.

In order to determine if the drugs directly affected cAMP, the perfusion solutions were tested in a cAMP-dependent, calmodulin-independent system. cAMP-dependent protein kinase was partially purified from the parotid glands of the rat and its activity was assayed by a modification of the method of Livesey *et al.* (12). cAMP-stimulated protein kinase activity was determined in the presence of the same concentrations of the drugs as used in the microperfusion studies.

Results. The concentration of calmodulin was determined in homogenates of kidney cortex and in brush border membranes prepared from these homogenates. The calmodulin concentration in the whole homogenates averaged 60.1 ± 17.1 ng/mg protein (n = 3); the calmodulin concentration in the brush border membranes averaged 44.3 ± 18.8 ng/mg protein (n = 3). The ratio of calmodulin concentration in the brush border membranes as compared to the whole homogenate was 0.73.

Under control conditions, water absorption averaged 2.46 ± 0.11 nl min^{-1} mm^{-1} (Table

TABLE I. WATER AND PHOSPHATE ABSORPTION IN THE PROXIMAL TUBULE OF THE RAT

Perfusion solution	n	L (mm)	PR (nl min^{-1})	J_v (nl min^{-1} mm^{-1})	J_{PO_4-2} (pmole min^{-1} mm^{-1})
Control	19	1.3 ± 0.1	18.7 ± 0.4	2.46 ± 0.11	10.7 ± 0.8
cAMP	15	1.5 ± 0.1	18.6 ± 0.6	1.61 ± 0.12*	9.1 ± 0.6
cAMP + TFP	19	1.3 ± 0.1	18.5 ± 0.6	2.40 ± 0.09	11.3 ± 1.1
cAMP + TFP oxide	17	1.2 ± 0.1	18.3 ± 0.8	1.67 ± 0.09*	10.9 ± 0.8
cAMP + W-13	16	1.6 ± 0.2	18.4 ± 0.8	2.48 ± 0.21	9.4 ± 1.1
cAMP + W-12	16	1.5 ± 0.1	17.2 ± 1.0	1.88 ± 0.14*	10.8 ± 0.7
TFP	10	1.1 ± 0.1	18.5 ± 1.0	2.36 ± 0.07	12.6 ± 1.4
TFP oxide	11	1.5 ± 0.2	18.1 ± 1.0	2.39 ± 0.17	10.8 ± 1.1
W-13	12	1.5 ± 0.2	17.5 ± 1.0	2.55 ± 0.17	12.8 ± 1.5
W-12	14	1.5 ± 0.1	17.2 ± 0.6	2.67 ± 0.26	11.02 ± 1.3

Note. Values are expressed as the means ± SEM n = number of tubules; L = length of perfused tubule; PR = perfusion rate; J_v = water absorption; and J_{PO_4-2} = phosphate absorption. *$P < 0.02$ as compared to controls.

I). The addition of 10^{-6} *M* cAMP to the perfusion solution resulted in a significantly lower rate of water absorption of 1.61 ± 0.12 nl min^{-1} mm^{-1}. The addition of either TFP or W-13 to the microperfusion solution containing cAMP resulted in rates of water absorption of 2.40 ± 0.09 and 2.48 ± 0.21 nl min^{-1} mm^{-1}, respectively, values significantly higher than that obtained with cAMP alone and not significantly different from those of controls. By contrast, the addition of analogs of TFP and W-13 with lower affinities for binding of calmodulin (TFP oxide and W-12) to the microperfusion solution containing cAMP did not significantly affect the rates of absorption of water. TFP, TFP oxide, W-13, or W-12 when added to the control microperfusion solution not containing cAMP resulted in rates of water absorption not significantly different from those of controls.

The ability of cAMP to activate a protein kinase derived from the parotid gland of the rat was examined in the presence of the drugs in order to determine if the drugs were binding cAMP, inactivating cAMP, or otherwise inhibiting the action of cAMP on protein kinase. The activity of cAMP-dependent protein kinase, expressed as a percentage of activity stimulated by cAMP alone, as determined in three separate assays, did not differ by more than ±10% with the addition of any of the drugs.

Discussion. Recent reviews have cataloged the number of species and organs in which calmodulin has been identified (1, 2). In the present studies, a sensitive radioimmunoassay was utilized which confirms that calmodulin is present in the kidney (3–5). The mere presence of protein, however, allows no insight into the role of calmodulin in transport processes. In addition, the radioimmunoassay utilized measures total calmodulin and not calmodulin activity. In a variety of test systems, a putative role for calmodulin in physiologic processes has been deduced from the effects of drugs or agents which have affinity for calmodulin and block its activity. This strategy was employed in the present experiments.

TFP and W-13 have both been found to have binding affinities for calmodulin in other tissue systems (13–16). The concentration of these agents employed in the present studies approximates the IC_{50} determined in these other systems (13, 14). Both agents reversed the cAMP-induced inhibition of water absorption in the proximal convoluted tubule of the rat. These findings are consistent with the conclusion that calmodulin is required for the expression of cAMP-associated inhibition of water absorption. The validity of such a conclusion, however, must be tempered in view of the uncertainty regarding the specificity of the drugs. It has been suggested that TFP and perhaps other drugs thought to be calmodulin antagonists may directly affect other facets of cell function by a mechanism unrelated to calmodulin and that the effects of TFP cannot be taken, a priori, to indicate a calmodulin-mediated reaction (17, 18). These nonspecific effects of the calmodulin antagonists may relate to the hydrophobic properties of the drugs. It has been reported, for example, that TFP may limit mitochondrial energy production and inhibit Na^+–K^+ ATPase activity by calmodulin-independent mechanisms (17, 18). Several lines of evidence in the present studies would indicate that the effects of the drugs were not the result of such nonspecific effects. First, in the absence of cAMP none of the drugs exerted an effect on water absorption. Moreover, the absorption of phosphate was not influenced by the drugs. Thus, if TFP or W-13 were changing the characteristics of the membrane, limiting mitochondrial energy production, or decreasing Na^+–K^+ ATPase activity, it would be anticipated that some response would be evident even in the absence of cAMP in the luminal perfusate. Second, analogs of TFP and W-13 with lesser binding affinities for calmodulin had no effect on water absorption and did not reverse the cAMP-associated inhibition of water transport (15, 16). Finally, TFP and W-13 are structurally different from one another. Findings in the present study also indicate that the drugs themselves did not inactivate cAMP or prevent its binding to a receptor. While the above considerations are suggestive that the effects of TFP and W-13 are not a nonspecific response, the findings do not totally exclude such a possibility. In view of these uncertainties, the most cautious interpretation of the results of the present studies is that TFP and

W-13 block the cAMP-associated inhibition of water absorption. The findings of the present studies are consistent with but do not unequivocally prove that the cAMP-induced inhibition of water transport in this nephron segment involves an interaction with calmodulin.

Prior studies have demonstrated that parathyroid hormone or cAMP inhibits the transport of water and phosphate in the proximal tubule (19–21). Recent studies have indicated that PTH and cAMP inhibit the absorption of water by altering the activity of the sodium-proton counter exchanger located in the brush border membrane of the proximal convoluted tubule (22, 23). In the present studies the transport of water but not phosphate was inhibited by cAMP. The reasons for these differences are unknown at present. It is possible then that the failure to discern a change in phosphate transport may reflect perfusion of segments of the proximal tubule in which phosphate transport is not influenced by cAMP (20, 24). Additional studies will be required to clarify this issue. Thus, while a role for calmodulin in the expression of the inhibitory effects of cAMP on the absorption of water is suggested from the results of the present experiments, no definitive statement can be made as to the possible role of calmodulin on the effect of cAMP on the transport of phosphate.

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