

# Effects of Osmolality and Oxygen Availability on Soluble Cyclic AMP-Dependent Protein Kinase Activity of Rat Renal Inner Medulla

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**ABSTRACT** The renal inner medulla is ordinarily exposed to osmolalities that are much higher and to O<sub>2</sub> tensions that are lower than those in other tissues. The effects of media osmolality and O<sub>2</sub> availability on basal and arginine vasopressin (AVP)-responsive soluble cyclic (c)AMP-dependent protein kinase activity were examined in slices of rat inner medulla. Increasing total media osmolality from 305 to 750 or 1,650 mosM by addition of urea plus NaCl to standard Krebs-Ringer bicarbonate buffer significantly reduced basal cAMP content and protein kinase activity ratios. This occurred in the presence or absence of O<sub>2</sub>. Incubation of slices in high osmolality buffer also blunted increases in inner medullary slice cAMP and protein kinase activity ratios induced by O<sub>2</sub>. These changes reflected predominantly an action of the urea rather than the NaCl content of high osmolality buffers. In contrast to effects on basal activity, high media osmolality significantly enhanced activation of inner medullary protein kinase by AVP. Conversely, increases in media O<sub>2</sub> content suppressed AVP stimulation of enzyme activity. This inhibitory effect of O<sub>2</sub> was best expressed at low osmolality. Naproxen and ibuprofen, inhibitors of prostaglandin biosynthesis, reduced basal kinase activity ratios and increased AVP responsiveness in the presence, but not in the absence, of O<sub>2</sub>. Exogenous prostaglandins (PG) modestly increased (PGE<sub>2</sub> and PGE<sub>1</sub>) or did not change (PGF<sub>2α</sub>) cAMP and protein kinase activity ratios in O<sub>2</sub>-deprived inner medullary slices. Protein kinase activation by PGE<sub>2</sub> was not observed in oxygenated inner medulla with high basal activity ratios. The stimulatory effects of PGE<sub>2</sub> and PGE<sub>1</sub> on protein kinase activity observed in O<sub>2</sub>-deprived slices were additive with those of submaximal or maximal AVP. PGE<sub>2</sub>, PGE<sub>1</sub>, and PGF<sub>2α</sub> all failed to suppress AVP activation

of protein kinase. Thus, enhanced endogenous PGE production may contribute to the higher basal protein kinase activity ratios induced by O<sub>2</sub>. However, the results do not support a role for PGE<sub>2</sub>, PGE<sub>1</sub>, or PGF<sub>2α</sub> in O<sub>2</sub>-mediated inhibition of AVP responsiveness. The present data indicate that both solute content and O<sub>2</sub> availability can alter the expression of AVP action on cAMP-dependent protein kinase activity in inner medulla. AVP activation of protein kinase is best expressed when osmolality is high and O<sub>2</sub> availability is low, conditions that pertain in inner medulla during hydropenia.

## INTRODUCTION

Under physiologic conditions the inner medulla of the kidney is routinely exposed to osmolalities that are much higher and to oxygen tensions that are much lower than those that normally pertain in other mammalian tissues (1-3). The potentially important influence of these unique conditions has not always been considered in studies of inner medullary metabolism. Recent observations suggest that both oxygen availability and solute concentration significantly alter inner medullary cyclic (c)AMP<sup>1</sup> economy (4-6). These same factors may also be determinants of the expression of the cellular actions of cAMP in inner medulla. Current evidence indicates that the biologic actions of cAMP in mammalian cells are transmitted through phosphorylation reactions mediated by cAMP-dependent protein kinases (7). cAMP-responsive kinase activity is present in both the soluble and particulate fractions of kidney (8, 9) and other tissues (10, 11), but the properties and regulation of the soluble enzyme activity have been most extensively examined (12-15). Hormonal activa-

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<sup>1</sup>Abbreviations used in this paper: AVP, arginine vasopressin; cAMP, cyclic AMP; KRBG, Krebs Ringer bicarbonate-glucose buffer; PG, prostaglandin.

tion of soluble protein kinase has been demonstrated in intact cell preparations of both renal cortex (16, 17) and medulla (18). However, regulation of renal medullary protein kinase as a function of solute concentration and tissue oxygenation has not been examined. Accordingly, in the present study, we assessed the effects of media osmolality and O<sub>2</sub> availability on basal and arginine vasopressin (AVP)-responsive soluble cAMP-dependent protein kinase activity in slices of rat inner medulla.

## METHODS

**Preparation of tissue.** Male Sprague-Dawley rats (Zivic-Miller Laboratories, Inc., Pittsburgh, Pa.) weighing 300–350 g were fasted but allowed ad libitum water for 18 h before study. Rats were anesthetized with pentobarbital (5 mg/100 g body weight i.p.). The kidneys were excised, placed immediately on filter paper moistened with 0.85% NaCl and maintained at 0–4°C. The renal capsules were removed and slices of cortex outer and inner medulla were prepared manually with a Stadie-Riggs microtome as described previously in detail (5).

**Slice incubations.** To assess tissue cAMP content and protein kinase activity, inner medullary slices were incubated in 25-ml flasks containing 2 ml of standard Krebs-Ringer bicarbonate buffer (total osmolality, 305 mosM; total Na<sup>+</sup>, 145 mM; HCO<sub>3</sub><sup>-</sup>, 25 mM; Ca<sup>2+</sup>, 1.5 mM; Mg<sup>2+</sup>, 1.25 mM; pH 7.4) containing 5 mM glucose and 1 mg/ml of bovine serum albumin (standard Krebs-Ringer bicarbonate-glucose buffer [KRBC]), or in KRBC whose osmolality was increased by the addition of NaCl plus urea. Unless otherwise indicated, buffer with a total osmolality of 750 mosM contained 175 mM Na<sup>+</sup> (150 mM NaCl) and 380 mM urea. Buffer with a total osmolality of 1,650 mosM contained 365 mM Na<sup>+</sup> (340 mM NaCl) and 900 mM urea, whereas that with a total osmolality of 2,000 mosM contained 425 mM Na<sup>+</sup> (400 mM NaCl) and 1,130 mM urea. The latter two buffers were studied because the total osmolalities, sodium, and urea contents resembled those reported in rat inner medulla during water deprivation (1, 19–21). In some experiments, buffers were prepared to assess effects of urea and NaCl separately (see Results). Stock solutions of urea were adjusted to pH 7.4 before use. Final osmolality of each buffer was measured with a vapor pressure osmometer (model 5100, Wescor, Inc., Logan, Utah). Determined osmolalities were within 1% of calculated values. All stock buffer solutions were gassed for 10 min with 95% N<sub>2</sub> and 5% CO<sub>2</sub> and tightly stoppered. Slice incubations were conducted at 37°C in Dubnoff metabolic shakers agitated at 100 cycle/min. Independent of the buffer employed, all inner medullary slices were initially incubated for 15 min in 95% N<sub>2</sub> and 5% CO<sub>2</sub> with a continuous gassing system into flasks that were sealed with a rubber septum and vented through a 25-g needle. O<sub>2</sub> content of the incubates was then adjusted to the desired final levels indicated in Results by reequilibrating flasks for 20 min with a gas phase consisting of 0–95% O<sub>2</sub>, 5% CO<sub>2</sub>, balance N<sub>2</sub>. Addition of all test agents (10–50 μl) was made by injection through the rubber septum of the flask with a Hamilton syringe (Hamilton Co., Reno, Nev.). All slice incubations for determination of cAMP and soluble cAMP-dependent protein kinase activity were conducted in the absence of an inhibitor of cyclic nucleotide phosphodiesterase activity. To determine tissue cAMP content, a total of 40–50 mg inner medulla was present per flask, whereas for assay of protein kinase activity 20–25 mg was employed per flask. In preliminary studies, basal cAMP and protein

kinase activities were compared in slices equilibrated with a gas mixture containing, 0, 5, or 50% O<sub>2</sub> for a final 20 or 100 min. The comparisons were made in 305, 750, and 1,650 mosM buffer. The relative differences in cAMP and protein kinase observed as a function of osmolality and O<sub>2</sub> after a 20-min incubation (see Results) were still evident at 100 min. cAMP accumulation and protein kinase activity were also examined as a function of time after addition of AVP or prostaglandin (PG)E<sub>2</sub> in slices incubated in 750 or 1,650 mosM buffer over a gas phase O<sub>2</sub> concentration range of 0–50%. Under the conditions studied and without an inhibitor of phosphodiesterase activity, peak cAMP responses occurred at 2 min after addition of test agents. Although largely expressed by 2 min, protein kinase responses in some instances were slightly higher at 5 min. Accordingly, to approximate most accurately the time of peak response for each parameter, slices for cAMP and protein kinase were sampled at 2 and 5 min after hormone addition, respectively.

cAMP content of the tissue was extracted with hot sodium acetate, assayed by the protein binding method (5, 16, 22), and authenticity of the determinations verified as previously described (5, 16). Blotted slices were weighed before incubation. cAMP was routinely expressed as picomoles per milligram preincubation wet weight, corrected for recovery. In some experiments, tissue cAMP content was calculated on the basis of postincubation wet weight and protein content, as well as preincubation wet weight. In these studies, inner medullary slices (≅100 mg total per incubation flask) were carefully blotted, wrapped in a section of aluminum foil of predetermined weight and weighed on a Mettler analytical balance (Mettler Instrument Corp., Hightstown, N. J.) to an accuracy of 0.01 mg before incubation. The slices were then incubated under the various conditions of media osmolality and O<sub>2</sub> availability described in Results. At the end of the incubation slices were again blotted and placed in preweighed microvials (total capacity 0.3 ml) containing 0.1 ml of 50 mM sodium acetate buffer, pH 4.0 at room temperature. Buffer contained 2,000 cpm of [<sup>3</sup>H]cAMP to monitor recovery. Vials were tightly sealed with Teflon-lined screw caps and heated to 95°C for 3 min in a heating block. The transfer procedure required ≅10 s. After reequilibration to room temperature, the vials were again weighed on an analytical balance. No detectable change in weight occurred as a function of the heating procedure in sealed vials containing 0.1 ml buffer without tissue. Slices plus buffer were transferred from the micro-vials to grinding vessels with three 0.1-ml aliquots of fresh sodium acetate buffer as wash. Tissue was then extracted as previously described for determination of cAMP and protein content (5, 16). Protein was determined by the Lowry method (23).

For assay of protein kinase activity, inner medulla from a single incubate was immediately homogenized at 0–4°C in 400 μl of 5 mM KH<sub>2</sub>PO<sub>4</sub> buffer (pH 6.8) containing 2 mM EDTA, 0.5 mM 3-isobutyl-1-methylxanthine and, routinely, 0.15 M NaCl. Homogenates were centrifuged at 0–4°C for 20 min at 20,000 g in a Sorvall centrifuge (SS 34 rotor, Ivan Sorvall, Norwalk, Conn.). The supernatant fraction was assayed for protein kinase activity on the same day as the slice incubation.

**Assay of cAMP-dependent protein kinase activity.** The reaction mixture for the assay of protein kinase activity of inner medulla was similar to that previously employed for study of activity in renal cortex (16), and originally described by Corbin and Reimann (24). The enzyme assay mixture contained at final concentration 12 mM KH<sub>2</sub>PO<sub>4</sub> (pH 6.8), 0.01 M NaF, 4 mM MgCl<sub>2</sub>, 0.23 mM [<sup>γ</sup>-<sup>32</sup>P] ATP (2 × 10<sup>6</sup> cpm/assay), 6.7 mg/ml of calf thymus histone (type 11A) and, when present, 2 μM cAMP. Under the standard assay conditions the reaction

was linear with respect to time for 10 min and added protein from 5 to 30  $\mu\text{g}$  for the 20,000-g soluble fraction of inner medulla, with  $\approx 80\%$  of the total activity (plus cAMP) abolished by the addition to the kinase reaction mixture of 50  $\mu\text{g}$  of protein kinase inhibitor prepared from rabbit muscle. Kinase activity is expressed as picomoles of inorganic phosphate incorporated into histone per min per milligram protein. Enzyme activity ratios were calculated from the rates of histone phosphorylation observed in the absence and those obtained in the presence of 2  $\mu\text{M}$  cAMP in the reaction mixture.

Use of standard low ionic strength (5 mM)  $\text{KH}_2\text{PO}_4$  buffer for slice homogenization was associated with recovery of much less total kinase activity in the 20,000-g supernatant fractions of inner medullary slices with prior exposure to AVP ( $327 \pm 39$  pmol/min per mg protein) than in those of control slices ( $693 \pm 75$ ). Addition of 2  $\mu\text{M}$  exogenous cAMP to low ionic strength homogenates of control slices before centrifugation was similarly associated with marked loss of total soluble enzyme activity ( $269 \pm 28$ ). This same phenomenon has been observed after homogenization of other tissues in low ionic strength buffer (2, 16, 17) because of binding of the catalytic subunit to the particulate fraction. It is prevented by buffer of higher ionic strength (16, 17, 25). Consistent with these earlier findings, the addition of 0.15 M NaCl, 0.5 M NaCl, or 0.5 M NaCl plus 1 M urea to the homogenizing buffer completely abolished the loss of total kinase activity from supernatant fractions of AVP-stimulated inner medulla, and had only minor effects on the basal kinase activity ratios ( $-\text{cAMP}/+\text{cAMP}$ ) in supernatant fractions prepared from homogenates of control slices (5 mM  $\text{KH}_2\text{PO}_4$  buffer,  $0.15 \pm 0.02$ ; buffer + 0.15 M NaCl,  $0.26 \pm 0.03$ ; buffer + 0.5 M NaCl and 1 M urea,  $0.25 \pm 0.03$ ). Accordingly, phosphate buffer containing 0.15 M NaCl was employed routinely for homogenization of inner medullary slices.

Although absolute protein phosphorylation rates were reduced at high osmolality (2,000 mosM), protein kinase activity ratios of slices incubated in the presence or absence of AVP were not altered by the final osmolality of the enzyme assay mixture. Therefore, standard low ionic strength enzyme reaction mixtures were employed to obtain the highest absolute rates of histone phosphorylation.

**Assay of slice ATP content.** ATP content of incubated inner medullary slices was determined in neutralized 5% perchloric acid extracts by the luciferin-luciferase bioluminescent method as previously reported in detail (5, 16, 26). cAMP content of these same supernatant fractions was assayed as described above.

**Determination of buffer oxygen concentration.** Media  $\text{O}_2$  concentration was calculated from the capacity of solutions to support glucose oxidation in the presence of excess (5 mM) glucose as previously described (27). Briefly, KRBG (2 ml) of different osmolalities was incubated in the absence of tissue at 37°C in 25-ml flasks with gas mixtures containing 0–50%  $\text{O}_2$ , 5%  $\text{CO}_2$ , and balance  $\text{N}_2$ , as described above for slice incubates. After 30 min, 0.5–1.5 ml of media was withdrawn from the sealed flasks with a Hamilton syringe (Hamilton Co., Reno, Nev.) and injected below  $\text{N}_2$ -equilibrated mineral oil in a 3-ml spectrophotometric cuvette. Glucose oxidase (10 IU), horseradish peroxidase (2-purpurogallin units) and *o*-dianisidine (80  $\mu\text{g}$ ) were then injected with a Hamilton syringe into the aqueous phase of the cuvette in a volume of iso-osmolal-deoxygenated KRBG (0.5–1.5 ml) sufficient to bring the total volume of the aqueous phase to 2 ml. Cuvettes were then incubated for 30 min at 37°C and glucose oxidation assessed spectrophotometrically. Glucose standards were similarly incubated for 30 min at 37°C. Standard curves were linear from 0 to 0.25 mM glucose. Identical curves were ob-

tained conducting incubations in distilled water gassed with 100%  $\text{O}_2$ , or in 305, 750, or 1,650 mosM KRBG gassed with 95%  $\text{O}_2$ , 5%  $\text{CO}_2$ . Validity of the method of  $\text{O}_2$  determination was confirmed by the following. (a) In distilled water equilibrated with gas phases containing 5, 10, 20, or 50%  $\text{O}_2$  at 37°C, dissolved  $\text{O}_2$  concentrations were  $71 \pm 8$ ,  $123 \pm 15$ ,  $236 \pm 30$  and  $491 \pm 53$   $\mu\text{M}$ , respectively. These values are consistent with the known solubility coefficient of  $\text{O}_2$  in water at 37°C (28). (b) The  $\text{O}_2$  concentration of 1,650 mosM NaCl-urea solutions (pH 7.4) saturated with air were determined at 37°C by the method described above, and by the coupling of NADH+ oxidation with changes in  $\text{O}_2$  activity as monitored with a model 53,  $\text{O}_2$  electrode (Yellow Springs Instrument Co., Inc., Yellow Springs, Ohio) (29). These methods yielded dissolved  $\text{O}_2$  concentrations of  $224 \pm 31$  and  $217 \pm 25$   $\mu\text{M}$ , respectively (mean  $\pm$  SE of determinations on three separate solutions).

Statistical significance of differences between mean values was assessed with Student's *t* test for unpaired values (30), unless otherwise indicated. The values shown represent the mean  $\pm$  SE of individual determinations derived from three to five separate experiments. In each individual experiment, slices from a group of rat kidneys were pooled and then randomly distributed among the various test conditions. Each incubation condition in a given experiment was represented by duplicate slices for cAMP and protein kinase. For purposes of statistical analysis, the average value derived from duplicate slices from the same experiment was entered as only a single value. Thus the total *n* was 6–10 (degrees of freedom 4–8) comparing 3–5 values of any two experimental populations to each other by Student's *t* test for unpaired values (30).

**Materials.** Protein kinase inhibitor (prepared from rabbit muscle), urea, and synthetic AVP (grade VI, 367 IU/mg) were purchased from Sigma Chemical Co., St. Louis, Mo. Gases were obtained from Liquid Carbonic Corp., Pittsburgh, Pa. and contents certified to be within 0.001–0.0001% of indicated values. Naproxen was generously supplied by Syntex Laboratories, Inc., Palo Alto, Calif. Ibuprofen and prostaglandins were provided by the Upjohn Co., Kalamazoo, Mich. Micro-vials (0.3 ml) were obtained from Fisher Scientific Co., Pittsburgh, Pa. Sources of all other materials have been previously reported (5, 16, 26).

## RESULTS

**Effects of media osmolality and  $\text{O}_2$  availability on basal protein kinase activity ratios of inner medullary slices.** Table I shows alterations in cAMP content and protein kinase activity ratios in rat inner medullary slices as a function of the osmolality of the incubation media and  $\text{O}_2$  content of the gas phase. In oxygenated slices, cAMP levels and kinase activity ratios of inner medulla fell progressively as media osmolality was increased from 305 to 1,650 mosM. A further increase in buffer osmolality to 2,000 mosM produced no additional decline in cAMP or in kinase activity ratios. In the absence of  $\text{O}_2$ , tissue cAMP and kinase activity ratios fell as media osmolality was increased from 305 to 750 mosM (Table I), but were not altered by a further increase in osmolality. As also shown in Table I, increases in cAMP and protein kinase activity ratios in response to alterations in the  $\text{O}_2$  content of the gas phase were clearly blunted as the osmolality of the incubation media was raised. At the higher media

TABLE I  
Effects of Osmolality and Oxygen Availability on cAMP Content and Activity Ratios of Soluble cAMP-Dependent Protein Kinase of Inner Medulla

	O <sub>2</sub> Content of gas phase	Osmolality of incubation media			
		305 (4)	750 (5)	1,650 (5)	2,000 (3)
	%	pmol/mg wet weight			
cAMP	0	1.34±0.17	0.58±0.07‡	0.31±0.04§	0.25±0.04
	5	1.91±0.25*	0.96±0.12*‡	0.28±0.04§	0.29±0.04
	10	2.84±0.33*	1.83±0.26*‡	0.39±0.05§	0.32±0.04
	20	4.26±0.51*	3.19±0.44*‡	0.75±0.08*§	0.64±0.07*
	50	5.73±0.68*	3.34±0.45*‡	0.69±0.08*§	0.71±0.08*
Protein kinase activity ratios	0	0.50±0.06	0.31±0.04‡	0.29±0.04	0.26±0.04
	5	0.68±0.07*	0.46±0.06*‡	0.28±0.04§	0.27±0.04
	10	0.73±0.08*	0.59±0.06*	0.31±0.04§	0.30±0.04
	20	0.82±0.09*	0.65±0.08*	0.44±0.05*§	0.43±0.05*
	50	0.89±0.10*	0.68±0.08*	0.47±0.05*§	0.45±0.05*

All inner medullary slices were initially incubated for 15 min at 37°C in 95% N<sub>2</sub>, 5% CO<sub>2</sub>. The buffer employed was either standard KRBG (305 mosM) or KRBG whose osmolality was adjusted to the higher value shown by addition of NaCl plus urea. After the preincubation period, incubates were equilibrated for a final 20-min period with a gas phase whose O<sub>2</sub> content is shown. At the conclusion of this final incubation period, some slices were extracted for cAMP content whereas others were homogenized and fractionated in phosphate buffer for subsequent assay of soluble protein kinase activity. Under the various slice-incubation conditions shown, total kinase activity (+cAMP in the kinase assay) present in the soluble fraction was comparable (range, 681±77 to 754±86 pmol/min per mg protein), and thus changes in kinase activity ratios (-cAMP/+cAMP) reflect changes in -cAMP activity. Number of separate experiments performed at each osmolality is indicated in parentheses.

\*  $P < 0.01$  versus value in the absence of O<sub>2</sub> at the same osmolality.

‡  $P < 0.01$  comparing value at 750 mosM to that at 305 mosM with the same gas phase O<sub>2</sub>.

§  $P < 0.01$  comparing value at 1,650 mosM to that at 750 mosM with the same gas phase O<sub>2</sub> (total  $n = 6-10$ , comparing groups by Student's  $t$  test for unpaired values).

osmolalities (1,650 or 2,000 mosM) 20% O<sub>2</sub> was required to increase cAMP and protein kinase activity ratios significantly over those observed in the absence of O<sub>2</sub>. By contrast, at the lower osmolalities (305 and 750 mosM) increases were detectable when the O<sub>2</sub> content of the gas phase was increased from 0 to 5%.

Table II compares the cAMP content of inner medullary slices, incubated at different media osmolalities and gas phase O<sub>2</sub> concentrations, using the postincubation wet weight and protein content as well as preincubation weight for reference indices. Over the range of O<sub>2</sub> concentration examined, changes in slice weight as a result of incubation were +10.9±2.4% (mean±SE of post-minus preincubation weight × 100/preincubation weight) at 305 mosM -9.2±2.4% at 750 mosM and -24.1±3.5% at 1,650 mosM. These differences are reflected by small reciprocal changes in calculated cAMP content when postincubation, rather than preincubation, weight is employed as the reference index (Table II). The changes in slice weight observed after exposure to 750 or 1,650 mosM buffer are similar in

magnitude to the changes in weight and estimated cell volume previously reported by Law in inner medulla slices from hydrated rats that have been incubated in buffers of comparable osmolality and composition (19, 31). As shown in Table II the differences in tissue cAMP content observed as a function of media osmolality and gas phase O<sub>2</sub> concentration expressing values on the basis of preincubation weight were still evident when either postincubation weight or tissue protein content was used as the reference index. Thus, the influence of osmolality and O<sub>2</sub> on tissue cAMP accumulation is reliably reflected by any of these reference indices. The modest alterations in weight, and presumably cell volume (19, 31), that occur as a result of exposure of inner medulla to different osmolalities and O<sub>2</sub> tensions do influence the precise quantitation of the comparisons of cAMP (Table II). However, they are insufficient to account for the changes in cAMP observed under different conditions of incubation.

ATP content of inner medulla fell as media os-

TABLE II  
Effects of Media Osmolality and O<sub>2</sub> Availability on Tissue cAMP Content Expressed as a Function of Pre- or Postincubation Wet Weight or Protein Content

O <sub>2</sub> Content of gas phase	Osmolality of incubation media (mosM)								
	305		750		1,650		305	750	1,650
	Initial	Final	Initial	Final	Initial	Final			
%	<i>pmol cAMP/mg wet wt</i>						<i>pmol cAMP/mg protein</i>		
0	1.53 ±0.18	1.32 ±0.15	0.61‡ ±0.08	0.65‡ ±0.08	0.29§ ±0.04	0.36§ ±0.05	19 ±2	6.6‡ ±0.7	4.1§ ±0.4
10	3.11* ±0.34	2.85* ±0.31	1.94*‡ ±0.23	2.16*‡ ±0.28	0.37§ ±0.05	0.45§ ±0.05	32* ±4	23*‡ ±3	4.9§ ±0.5
50	5.96* ±0.62	5.37* ±0.59	3.18*‡ ±0.47	3.52*‡ ±0.48	0.73*§ ±0.09	0.91*§ ±0.11	58* ±6	41*‡ ±5	9.4*§ ±1

Slices were incubated as described in Table I. Pre- and postincubation wet weights of each slice were determined on an analytical balance. After postincubation weighing, slices were extracted for cAMP and protein content. Each value represents the mean ± SE of determinations from three separate experiments.

\*  $P < 0.01$  versus value in the absence of O<sub>2</sub> at the same osmolality.

‡  $P < 0.01$  comparing value at 750 mosM to that at 305 mosM with the same gas phase O<sub>2</sub>.

§  $P < 0.01$  comparing value at 1,650 mosM to that at 750 mosM with the same gas phase O<sub>2</sub>.

molality was increased, and this occurred in the presence or absence of O<sub>2</sub>. In O<sub>2</sub>-deprived inner medulla, ATP was  $0.37 \pm 0.04$   $\mu\text{mol/g}$  wet wt in 305 mosM buffer and  $0.19 \pm 0.02$  in 1,650 mosM buffer, with concomitant cAMP levels of  $1.52 \pm 0.19$  and  $0.25 \pm 0.04$  pmol/mg wet wt, respectively ( $n = 6$ ,  $P < 0.01$  comparing values at the high and low osmolality). In the presence of 50% O<sub>2</sub>, ATP was  $0.84 \pm 0.10$   $\mu\text{mol/g}$  wet wt at 305 mosM and  $0.43 \pm 0.05$  at 1,650 mosM, with corresponding cAMP levels of  $5.16 \pm 0.71$  and  $0.56 \pm 0.07$  pmol/mg wet wt, respectively. Thus, reduced tissue ATP content at high osmolality may have contributed to the decline in cAMP observed. However, cAMP was much higher in slices incubated anaerobically at 305 mosM than in those incubated with 50% O<sub>2</sub> at 1,650 mosM, whereas ATP levels were comparable under these two incubation conditions. Accordingly, alterations in total tissue ATP content do not adequately account for the changes in cAMP observed.

As shown in Table III, media O<sub>2</sub> concentrations varied as a function of gas phase O<sub>2</sub> content (0–50%), but did not change significantly as a function of media osmolality over a range of 305–1,650 mosM. The relatively constant concentration of dissolved O<sub>2</sub> observed over this relatively narrow range of media osmolality (Table II) is consistent with previous findings (<15% change) using a combination of NADH+ oxidation and an O<sub>2</sub> electrode to estimate O<sub>2</sub> concentration (30). Thus, reduced O<sub>2</sub> solubility did not account for the blunted cAMP and protein kinase responses to O<sub>2</sub> over the range of O<sub>2</sub> and media osmolality examined (Tables I, II).

Table IV shows the effects of separate additions of urea or NaCl to the slice incubation media on the cAMP content and protein kinase activity ratios of inner medulla incubated with 20% O<sub>2</sub>. When the total osmolality of the incubation media was raised from 305 to 1,650 mosM by addition of urea alone, basal cAMP and kinase activity ratios were comparable to those observed in slices incubated in 1,650 mosM media containing 365 mM Na<sup>+</sup> plus 900 mM urea. By contrast, excess NaCl was relatively ineffective. cAMP content of slices incubated in 1,650 mosM buffer containing only excess NaCl was lower than that observed in standard 305 mosM buffer, but kinase activity ratios

TABLE III  
Alterations in Media Oxygen Concentration with Changes in Media Osmolality and Gas Phase Oxygen Content

Gas phase O <sub>2</sub> content	Media osmolality*		
	305	750	1,650
	$\mu\text{M O}_2$		
0	0–2	0–3	0–2
5	78 ± 9	86 ± 9	83 ± 8
10	140 ± 16	158 ± 18	152 ± 16
20	223 ± 27	216 ± 24	197 ± 21
50	472 ± 50	434 ± 47	469 ± 52

O<sub>2</sub> concentrations of solutions were calculated from their capacity to support glucose oxidation in the presence of excess (5 mM) glucose as described in Methods. Each value represents the mean ± SE or range (no O<sub>2</sub>) of determinations from three separate experiments.

\* Media osmolality is measured in milliosmoles per liter.

TABLE IV  
Comparison of the Effectiveness of NaCl vs. Urea in Reduction of Inner Medullary cAMP and Protein Kinase Activity Ratios

Buffer		Additions to 305 mosM buffer		cAMP	Protein kinase activity ratios
Total osmolality	Total Na <sup>+</sup>	Urea	NaCl		
<i>mosM</i>	<i>mM</i>	<i>mmol/liter</i>		<i>pmol/mg wet wt</i>	<i>-cAMP/+cAMP</i>
305	145	0	0	4.36±0.52	0.82±0.09
1,650	365	900	220	0.85±0.09*	0.34±0.04*
1,650	145	1,345	0	0.72±0.08*	0.31±0.04*
1,650	817	0	672	2.58±0.32*†	0.75±0.09‡
1,205	145	900	0	1.35±0.16*	0.54±0.06*
1,815	900	0	755	2.21±0.29*§	0.73±0.08§

Slices were incubated in standard KRBG (305 mosM, 145 mM Na<sup>+</sup>) or in KRBG whose total osmolality and Na<sup>+</sup> content was increased to the final values shown by additions of the quantities of urea or NaCl indicated. Slices were initially incubated in 95% N<sub>2</sub>, 5% CO<sub>2</sub> for 15 min, and then in 20% O<sub>2</sub>, 5% CO<sub>2</sub>, 75% N<sub>2</sub> for 20 min. Each value represents the mean±SE of determinations pooled from three separate experiments.

\* *P* < 0.01 compared to value for 305 mosM buffer.

† *P* < 0.01 comparing 1,650 mosM with no added urea to 1,650 mosM buffers containing urea.

§ *P* < 0.01 comparing 900 mM total Na<sup>+</sup> to 900 mM urea in buffers of different total osmolalities (total *n* = 6, comparing groups by Student's *t* test for unpaired values).

were not detectably different under these two conditions. Furthermore, in either iso-osmolal (1,650 mosM) or hyperosmolal (1,815 mosM) media containing excess NaCl without urea, tissue cAMP and kinase activity ratios were significantly higher than those observed in 1,650 mosM buffers that contained urea (Table IV). These observations indicate a major and essential role for urea in the marked reduction of protein kinase activity ratios observed in inner medullary slices at high osmolality (Table I). However, in the presence of urea, media NaCl content, total osmolality, or both also contributed to the changes observed (Table IV).

**Effect of media osmolality and O<sub>2</sub> availability on AVP-responsive protein kinase activity.** Fig. 1 compares the effects of AVP on cAMP and protein kinase activity ratios in slices incubated in buffer of 750 or 1,650 mosM with a gas phase containing 5% O<sub>2</sub>. At the higher media osmolality, basal cAMP and protein kinase activity ratios were reduced and sensitivity of these parameters to AVP stimulation was enhanced. The concentration of AVP yielding approximately half-maximal stimulation of cAMP was 0.2 nM at 1,650 mosM and 10 nM at 750 mosM. Similarly, for protein kinase 20 pM AVP gave half-maximal activation

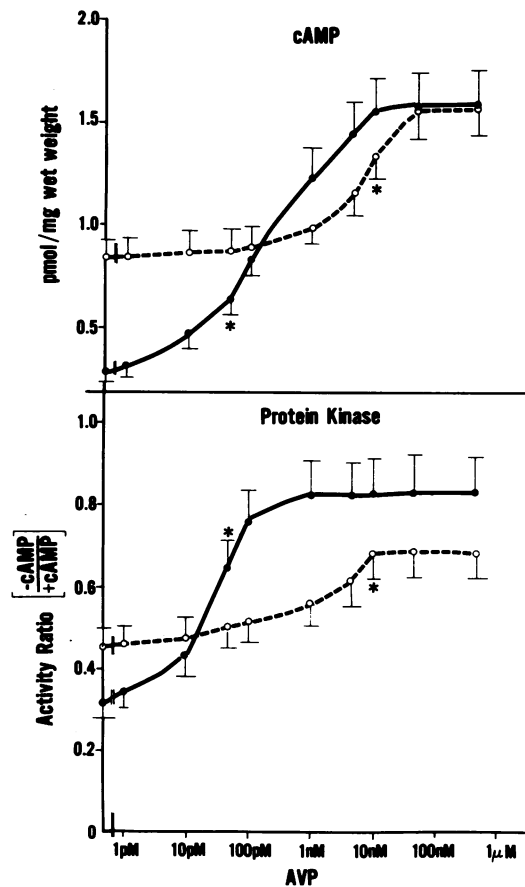


FIGURE 1 Effects of the osmolality of slice incubation media on alterations in inner medullary cAMP and protein kinase activity ratios mediated by AVP. Slices were incubated at 37°C in KRBG with total osmolalities of 750 mosM (○) or 1,650 mosM (●). The gas phase consisted of 95% N<sub>2</sub>, 5% CO<sub>2</sub> for the first 15 min of the incubation, and 5% O<sub>2</sub>, 5% CO<sub>2</sub>, and 90% N<sub>2</sub> for the final 20 min. AVP was present at the concentrations shown for the final 2 min (cAMP) or 5 min (protein kinase) of the incubation. Total kinase activities (+2 μM cAMP) under the different incubation conditions shown were comparable (range, 703±85 to 786±89 pmol/min per mg protein). Each value represents the mean±SE of individual determinations from 4 (cAMP) or 5 (protein kinase) separate experiments. The asterisk (\*) indicates the lowest concentration of AVP yielding significant stimulation of cAMP or kinase activity ratios compared to the value without AVP at the same osmolality (*P* < 0.01; total *n* = 8 for cAMP and 10 for protein kinase).

at 1,650 mosM whereas 5 nM AVP was required at 750 mosM. In slices incubated in 305 mosM buffer and 5% O<sub>2</sub> without an inhibitor of cyclic nucleotide phosphodiesterase activity, increases in cAMP or protein kinase activity were not detectable in response to concentrations of AVP up to 0.1 μM (not shown). Absolute cAMP levels in the presence of maximally effective concentrations of AVP did not differ in slices incubated in 750 or 1,650 mosM media (Fig. 1). This

was the case when cAMP values were expressed on the basis of postincubation wet weight ( $2.15 \pm 0.29$  pmol/mg at 750 mosM vs.  $2.52 \pm 0.33$  at 1,650 mosM) or protein content ( $26 \pm 2$  pmol/mg at 750 mosM vs.  $29 \pm 2$  at 1,650 mosM), as well as preincubation wet weight (Fig. 1). Therefore, enhancement of cAMP responsiveness to AVP was relative and a function of lower basal cAMP at high osmolality. By contrast, the protein kinase activity ratios in the presence of 30 nM AVP (Fig. 1) were absolutely lower in 750 mosM buffer ( $0.67 \pm 0.08$ ) than in 1,650 mosM buffer ( $0.83 \pm 0.10$ ,  $P < 0.01$ ,  $n = 5$  by paired analysis) despite a higher basal kinase activity ratio at the low osmolality. Thus, under two distinct incubation conditions, similar total tissue cAMP was associated with detectably different kinase activity ratios. Nevertheless, under both incubation conditions a significant increment in cAMP induced by AVP was associated with an increase in the protein kinase activity ratio (Fig. 1). To assess the possibility that the changes in the protein kinase activity ratio observed in AVP-treated slices reflected events occurring after tissue disruption due to release of cAMP, charcoal (10 mg/ml) was added to the buffer

used for homogenization as previously described (16). The presence of charcoal did not influence the increases in kinase activity ratios observed in slices that had been incubated with 30 nM AVP, but completely abolished enzyme activation induced in homogenates of control slices by addition of  $2 \mu\text{M}$  exogenous cAMP. These findings suggest that the AVP-related changes in kinase activity ratios occurred before tissue homogenization. They are analogous to results described earlier in PTH-stimulated renal cortical slices (16).

Table V compares AVP responses of inner medullary slices incubated in either 750 or 1,650 mosM buffer as a function of the  $\text{O}_2$  content of the gas phase. In the absence of  $\text{O}_2$ , slices incubated in 750 mosM had basal cAMP levels that were higher, but basal protein kinase activities that were similar, to those of slices incubated in 1,650 mosM buffer. A cAMP response to a submaximal concentration of AVP (50 pM) was detectable only at the higher osmolality (Table V). Increases in protein kinase activity in response to both maximal and submaximal AVP were clearly blunted in  $\text{O}_2$ -deprived slices incubated in 750 mosM compared to 1,650 mosM buffer, results which imply an  $\text{O}_2$ -

TABLE V  
Effects of Oxygen on the Responsiveness of Soluble cAMP-Dependent Protein Kinase Activity of Inner Medulla to Vasopressin

Slice incubation conditions			Protein kinase activity			
Buffer	$\text{O}_2$ content of gas phase	Additions	cAMP	-cAMP	+cAMP	-cAMP/+cAMP
mosM	%		pmol/mg wet wt	pmol/min/mg protein		
750	0	None	$0.64 \pm 0.08$	$215 \pm 27$	$742 \pm 83$	$0.29 \pm 0.04$
		50 pM AVP	$0.71 \pm 0.08$	$259 \pm 30$	$786 \pm 86$	$0.33 \pm 0.04$
		30 nM AVP	$1.87 \pm 0.23^*$	$436 \pm 51^*$	$716 \pm 79$	$0.61 \pm 0.08^*$
	10	None	$1.49 \pm 0.18 \ddagger$	$389 \pm 45 \ddagger$	$723 \pm 81$	$0.54 \pm 0.06 \ddagger$
		30 nM AVP	$2.06 \pm 0.27^*$	$410 \pm 48$	$695 \pm 76$	$0.59 \pm 0.07$
	50	None	$3.25 \pm 0.40 \ddagger$	$442 \pm 53 \ddagger$	$702 \pm 78$	$0.63 \pm 0.08 \ddagger$
30 nM AVP		$3.42 \pm 0.39 \ddagger$	$451 \pm 52$	$754 \pm 83$	$0.60 \pm 0.07$	
1,650	0	None	$0.32 \pm 0.04$	$184 \pm 22$	$683 \pm 76$	$0.27 \pm 0.04$
		50 pM AVP	$0.59 \pm 0.07^*$	$412 \pm 46^*$	$749 \pm 84$	$0.55 \pm 0.07^*$
		30 nM AVP	$1.75 \pm 0.21^*$	$647 \pm 68^*$	$732 \pm 80$	$0.86 \pm 0.09^*$
	10	None	$0.35 \pm 0.04$	$228 \pm 31$	$738 \pm 82$	$0.31 \pm 0.04$
		30 nM AVP	$1.91 \pm 0.25^*$	$643 \pm 75^*$	$775 \pm 89$	$0.83 \pm 0.09^*$
	50	None	$0.84 \pm 0.11 \ddagger$	$335 \pm 38 \ddagger$	$713 \pm 74$	$0.47 \pm 0.05 \ddagger$
		30 nM AVP	$1.86 \pm 0.23^*$	$475 \pm 50^* \ddagger$	$726 \pm 79$	$0.63 \pm 0.07^* \ddagger$

All slices were initially incubated at  $37^\circ\text{C}$  for 15 min in the absence of  $\text{O}_2$ , and then for an additional 20 min with the gas phase  $\text{O}_2$  shown. AVP was present for the final 2 min (cAMP) or 5 min (protein kinase) where indicated. Each value represents the mean  $\pm$  SE of determinations pooled from four separate experiments.

\*  $P < 0.01$  compared to value with no added AVP under the same incubation conditions.

‡  $P < 0.01$  compared to corresponding value in the absence of  $\text{O}_2$  at the same buffer osmolality (total  $n = 8$ , comparing groups by Student's  $t$  test for unpaired values).

independent influence of osmolality on expression of this action of AVP. In 1,650 mosM buffer, absolute cAMP accumulation in the presence of 30 nM AVP was not measurably different at 0, 10, or 50% O<sub>2</sub>. Only the relative cAMP response to AVP (AVP/basal) was reduced at 50% O<sub>2</sub>, as a result of the higher basal cAMP under these conditions. By contrast, both the absolute and the relative increases in protein kinase activity induced by AVP were suppressed in slices incubated at 1,650 mosM with 50% compared to 0 or 10% O<sub>2</sub>. In slices exposed to 750 mosM buffer, O<sub>2</sub> had analogous suppressive effects on the protein kinase responses of inner medulla to AVP. However, at this lower media osmolality, inhibitory effects of O<sub>2</sub> on AVP activation of protein kinase were evident at a gas phase O<sub>2</sub> content of 10% (Table V).

*Effects of PG synthetic inhibitors and exogenous PG on AVP-responsive protein kinase activity.* Naproxen, an inhibitor of PG synthesis which is known to be effective in renal medulla (32–34), significantly lowered basal cAMP and protein kinase activity ratios of inner medullary slices incubated with 50% O<sub>2</sub> in 1,650 mosM buffer (Table VI). The drug had no effect on these parameters in slices incubated without O<sub>2</sub>, and did not interfere directly with assays of cAMP or protein kinase activity. In the presence of 50% O<sub>2</sub>, 0.5 nM AVP failed to increase inner medullary cAMP or protein kinase. However, this concentration of AVP was effective in the absence of O<sub>2</sub>, or at 50% O<sub>2</sub> in the presence of naproxen (Table VI). Ibuprofen, 75 µg/ml, had effects similar to those of naproxen. Thus, ibuprofen lowered by 50–60% the basal cAMP level of inner medulla incubated in 1,650 mosmol buffer at an O<sub>2</sub> concentration of 50%, but had no detectable effect on absolute cAMP accumulation in the presence of 0.5 nM AVP. Basal kinase activity ratios of oxygenated inner medulla were 0.40±0.05 and 0.28±0.04 with and without ibuprofen, respectively. By contrast, in the presence of 0.5 nM AVP, the kinase activity ratios were 0.47±0.05 with and 0.64±0.07 without ibuprofen.

Table VII shows the effects of PGE<sub>2</sub> on inner medullary cAMP and protein kinase activity ratios after addition of this agent alone or in combination with AVP. Slices in these experiments were deprived of O<sub>2</sub> throughout to suppress endogenous synthesis of PG (6, 32). Under these conditions, increases in cAMP but not in protein kinase activity ratios were detected in response to 0.1 µM PGE<sub>2</sub>. The increase in cAMP mediated by 0.1 µM PGE<sub>2</sub> was similar to that observed with 50 pM AVP, but only the latter agent detectably increased kinase activity ratios. Although both cAMP and kinase activity ratios were increased by 0.1 nM PGE<sub>2</sub>, changes in kinase activity were relatively small (Table VII). When the gas phase O<sub>2</sub> content was increased to 50%, basal cAMP and kinase activity ratios rose, and responses of both parameters to 0.1 mM PGE<sub>2</sub>

TABLE VI  
*Effects of Naproxen on Protein Kinase Activation by Vasopressin in Inner Medullary Slices Incubated at High Osmolality in the Presence and Absence of Oxygen*

Additions	Gas phase		Protein kinase activity ratio
	% O <sub>2</sub>	cAMP pmol/mg wet wt	
None	0	0.26±0.03	0.28±0.04
75 µg/ml naproxen		0.33±0.04	0.31±0.04
0.5 nM AVP		1.35±0.17*	0.76±0.09*
Naproxen + AVP		1.22±0.14*	0.72±0.08*
None	50	0.93±0.11	0.45±0.06
Naproxen		0.38±0.05†	0.29±0.04†
AVP		1.20±0.15	0.49±0.06
Naproxen + AVP		1.29±0.18*	0.61±0.07*

All slices were incubated in 1,650 mosM buffer as outlined in Table III. Naproxen was added just before equilibration of incubates with final gas phase O<sub>2</sub> shown. AVP was present for the final 2 min (cAMP) or 5 min (protein kinase). Each value represents the mean±SE of determinations pooled from four separate experiments.

\*  $P < 0.01$  comparing value with AVP ( $\pm$ naproxen) to corresponding value without addition of hormone.

†  $P < 0.01$  comparing naproxen alone to value with no additions (total  $n = 8$ , comparing groups by Student's  $t$  test for unpaired values). Alteration in the protein kinase activity ratios shown reflect changes in the cAMP-independent activity ( $-$ cAMP in kinase assay).

were abolished (not shown). Combined addition of an ineffective or a maximal concentration of PGE<sub>2</sub> with a submaximal or maximal concentration of AVP resulted in increases in cAMP and protein kinase activity that were either not different (1.0 nM PGE<sub>2</sub>) or greater (0.1 mM PGE<sub>2</sub>) than those observed with AVP alone (Table VII). Combined addition of 0.1 mM PGE<sub>1</sub> with 0.5 nM AVP gave additive effects on cAMP and protein kinase, whereas 0.1 mM PGF<sub>2 $\alpha$</sub>  had no influence on basal levels of these parameters or their responses to AVP (not shown).

## DISCUSSION

The results of the present study clearly demonstrate that basal cAMP-dependent protein kinase activity is low in inner medullary slices exposed to high concentrations of urea and NaCl (Table I). Moreover, soluble protein kinase activity of inner medulla is most sensitive to stimulation by AVP in the presence of concentrations of these solutes (Fig. 1 and Table V) that pertain in inner medulla during hydropenia (1–3, 20, 35, 36). The effects of osmolality on protein kinase activity in inner medullary slices were expressed through processes both independent of, and interdependent with, the influence of O<sub>2</sub> availability. In



TABLE VII  
Effects of Vasopressin and PGE<sub>2</sub> on Inner Medullary cAMP  
Content and Protein Kinase Activity

Additions	Protein kinase activity ratio	
	cAMP pmol/mg wet wt	-cAMP/+cAMP
None	0.31±0.04	0.28±0.04
50 pM AVP	0.64±0.08*	0.56±0.07*
0.5 nM AVP	1.18±0.16*	0.71±0.08*
30 nM AVP	1.73±0.22*	0.84±0.09*
1.0 nM PGE <sub>2</sub>	0.28±0.04	0.29±0.04
0.1 μM PGE <sub>2</sub>	0.76±0.09*	0.33±0.04
0.1 mM PGE <sub>2</sub>	1.75±0.17*	0.45±0.05*
50 pM AVP		
+ 1.0 nM PGE <sub>2</sub>	0.68±0.08	0.51±0.07
+ 0.1 mM PGE <sub>2</sub>	1.69±0.19‡	0.76±0.09‡
0.5 nM AVP		
+ 1.0 nM PGE <sub>2</sub>	1.23±0.15	0.75±0.09
+ 0.1 mM PGE <sub>2</sub>	2.25±0.30‡	0.89±0.10
30 nM AVP		
+ 1.0 nM μM PGE <sub>2</sub>	1.78±0.24	0.81±0.09
+ 0.1 mM PGE <sub>2</sub>	2.79±0.35‡	0.97±0.11

Slices were incubated at 37°C in 1,650 mosM buffer without O<sub>2</sub> for 35 min. AVP and(or) PGE<sub>2</sub> were added at the final concentrations shown. The ethanol diluent of PGE<sub>2</sub> did not alone alter basal or AVP-responsive cAMP and protein kinase at the final concentration employed (0.25% vol/vol). Each value represents the mean±SE of determinations pooled from three separate experiments.

\*  $P < 0.01$  comparing values with AVP or PGE<sub>2</sub> alone to values with no addition of test agent.

‡  $P < 0.01$  comparing value with AVP plus PGE<sub>2</sub> to value with the same concentration of AVP alone (total  $n = 6$ , comparing groups by Student's  $t$  test for unpaired values).

general, basal cAMP and kinase activity ratios increased (Table I), and the relative (cAMP) or absolute (protein kinase) responses of these two parameters to AVP declined with a reduction in media osmolality, an increase in O<sub>2</sub> availability, or both (Table V). An oxygen-independent effect of osmolality is supported by the finding that changes in media osmolality altered basal cAMP and protein kinase activity ratios and the responses of these parameters to AVP in inner medulla deprived of O<sub>2</sub> (Tables I and V). A relationship may exist between the effects of osmolality to lower cAMP in intact inner medulla and the suppression of medullary adenylate cyclase activity observed by Dousa (4) in subcellular preparations incubated with high concentrations of NaCl and urea. However, this relationship remains uncertain because in the broken cell system (4) both basal and AVP-responsive adenylate cyclase activity were suppressed by high solute concentration. By contrast, whereas high osmolality clearly blunted the action of O<sub>2</sub> to increase cAMP and kinase activity ratios in slices (Table I), responses to

AVP were concomitantly enhanced by these conditions (Fig. 1 and Table V). The enhancement of AVP-induced cAMP accumulation at high osmolality and(or) low O<sub>2</sub> was relative, and a reflection of the lower basal cAMP levels under these conditions. Absolute cAMP accumulation in the presence of AVP did not differ detectably (Tables V–VII). By contrast, the potentiation of AVP effects on protein kinase activity ratios as a function of high osmolality and(or) low O<sub>2</sub> was absolute (Fig. 1, Table V–VII). Accordingly, depending upon the condition of incubation of inner medulla, significantly different protein kinase activity ratios were found in association with comparable levels of total tissue cAMP. A relationship between alterations in kinase activity ratios examined in tissue homogenates and intracellular events has not been established directly. Thus, some caution is warranted in correlating changes in activity ratios observed after tissue homogenization to total cellular cAMP levels. Nevertheless, the failure of added charcoal to influence the kinase activity ratios observed in homogenates of AVP-stimulated slices and the different activity ratios found in association with similar total tissue cAMP levels both support the conclusion that the changes in protein kinase activity ratios observed reflect intracellular events. The dissociation between total tissue cAMP and protein kinase activity ratios evident in inner medulla may be related to compartmentalization of cAMP and protein kinase (10). Inner medullary slices are composed of several cell types. Independent pools of cAMP within or among these cells may respond differently to changes in osmolality, O<sub>2</sub>, PG, or AVP.

Previous observations have indicated that the increase in inner medullary cAMP at high O<sub>2</sub> is related at least in part to enhanced endogenous synthesis of PG of the E type (5, 6), presumably PGE<sub>2</sub> (34, 37). Thus, indomethacin and meclofenamate, two structurally distinct inhibitors of PG biosynthesis (32), reduce basal cAMP of oxygenated inner medulla and permit expression of the action of exogenous PGE<sub>2</sub> to increase medullary cAMP at high O<sub>2</sub> tension (5). In the present study results obtained with naproxen and ibuprofen, other inhibitors of PG biosynthesis (32–34), were consistent with a role for endogenous PGE synthesis in the mediation of high basal protein kinase activity ratios seen in oxygenated inner medulla. Although several interpretations are possible, the effects of naproxen and ibuprofen also suggest that suppression of protein kinase responses to AVP in oxygenated tissue might be linked to enhanced endogenous PG synthesis (Table VI). These findings are compatible with *in vivo* actions of inhibitors of PG synthesis to potentiate the cAMP and hydro-osmotic responses of AVP (38, 39). However, when examined in O<sub>2</sub>-deprived slices whose endogenous PG synthesis was likely suppressed (6, 32), exogenous PGE<sub>2</sub>, PGE<sub>1</sub>, or PGF<sub>2α</sub> could

not be specifically implicated as inhibitors of AVP responsiveness. The increases in cAMP mediated by either PGE<sub>2</sub> or O<sub>2</sub> appeared to be less effective in the activation of soluble inner medullary protein kinase than were similar or smaller increases induced by AVP (Tables V and VII). As noted above, these results may reflect distinct AVP and PGE<sub>2</sub>-responsive pools of cAMP in inner medulla with differential influences on soluble protein kinase activity. Previous reports of additive stimulatory effects of AVP and PGE<sub>2</sub> on renal medullary adenylate cyclase activity (40, 41) support this suggestion. Although other explanations are not excluded, the potentiation of AVP action induced by naproxen in oxygenated inner medulla may be related to reduced generation of intermediates in the PG biosynthesis pathway rather than to formation of end products such as PGE<sub>2</sub>. In this regard, it is of some interest that the PG endoperoxide (PGH<sub>2</sub>) has been shown to inhibit hormone responsive adenylate cyclase in other systems (42, 43), and recently suppression of AVP-responsive adenylate cyclase by PGH<sub>2</sub> of rat inner medulla has also been described (44).

The present data provide no specific support for a relationship between endogenous PG synthesis and the modification of basal or AVP-responsive protein kinase activity induced by changes in media osmolality but do not exclude this possibility. A reduction in endogenous PG synthesis has been described in oxygenated inner medulla incubated in media containing high concentrations of both urea and NaCl (6), similar to those employed in the present study. By contrast, marked stimulation of PG synthesis was found after exposure of this same tissue to media containing high concentrations of NaCl alone (45). As illustrated by the very different actions of urea and NaCl on inner medullary kinase activity (Table IV), the specific solutes involved, as well as total osmolality, may be critical determinants of the PG synthetic responses encountered (6, 45). Consistent with this suggestion, a reciprocal relationship between inner medullary PG and sodium content has recently been reported in rat inner medulla *in vivo*, where the concentration of urea would also be high (46). In the current study, effects of osmolality on basal and AVP-responsive kinase activity ratios were expressed in O<sub>2</sub>-deprived inner medulla (Tables I and V). Similarly, naproxen failed to alter activity ratios in O<sub>2</sub>-deprived slices exposed to either 750 mosM (not shown) or 1,650 mosM buffer (Table VI). Because O<sub>2</sub> is required for PG synthesis (32), these findings suggest that the actions of osmolality on protein kinase activity in inner medulla may be, at least in part, independent of alterations in endogenous PG production induced by osmolality.

Finally, it should be emphasized that the very high basal cAMP levels and protein kinase activity ratios observed in rat inner medullary slices incubated in

standard KRBG (305 mosM) or exposed to a gas phase containing in excess of 5 or 10% O<sub>2</sub> may not have physiologic counterparts *in vivo*. Inner medullary O<sub>2</sub> tension seldom exceeds 60 mm Hg (2, 3) and osmolality in this region of the kidney is generally >500 mosM even during water diuresis (1, 19–21, 31). The routine use of standard low osmolality buffers and high ambient O<sub>2</sub> concentrations could artifactually increase basal kinase activity ratios and diminish the apparent sensitivity of this parameter to AVP stimulation *in vitro*. This is well demonstrated in the present study (Fig. 1, Tables I and V). At present, the physiologic implications of our observations can only be suggested. The decline in sensitivity of soluble protein kinase to AVP stimulation as a function of a fall in media urea and NaCl content or an increase in O<sub>2</sub> availability may be a factor in the reduced hydro-osmotic action of AVP seen *in vivo* under several conditions, including chronic water loading, volume expansion, or administration of osmotic diuretics (47–49). Current evidence indicates that in all of these circumstances medullary solute content *in vivo* is reduced (1, 19–21, 31, 50, 51), whereas vasa recta blood flow and presumably O<sub>2</sub> delivery to the inner medulla is increased (52, 53). These changes, in turn, may decrease the responsiveness of soluble cAMP-dependent protein kinase to AVP.

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