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Research Article

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Calcium-dependent Action of Osmolality on Adenosine 3′,5′-Monophosphate Accumulation in Rat Renal Inner Medulla

EVIDENCE FOR A RELATIONSHIP TO CALCIUM-RESPONSIVE ARACHIDONATE RELEASE AND PROSTAGLANDIN SYNTHESIS

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ABSTRACT When urea and NaCl are employed as the major solutes of high osmolality buffers, the cyclic AMP (cAMP) content of oxygenated slices of rat renal inner medulla increases three- to fivefold as osmolality is decreased from 1,650 to 305 mosM. Incubation of slices in Ca^{2+}-free media containing 2 mM EGTA largely abolished this action of osmolality on cAMP, whereas exclusion of Mg^{2+} or K^+ from the incubation media was without effect. Addition of Ca^{2+} to Ca^{2+}-deprived inner medulla incubated at 750 mosM (175 mM Na^+, 380 mM urea) significantly increased tissue cAMP and media prostaglandin (PG)E accumulation. Ca^{2+} also stimulated the release of ^{14}C-fatty acid from Ca^{2+}-deprived slices prelabeled with [^{14}C]arachidonate, but not from those labeled with [^{14}C]palmitate. The divalent cation ionophore A23187 enhanced the actions of Ca^{2+} to increase tissue cAMP, media PGE accumulation, and the release of [^{14}C]-arachidonate from prelabeled inner medulla. By contrast, when slices were incubated at 1,650 mosM (365 mM Na^+, 900 mM urea) in the presence or absence of A23187, all of these actions of Ca^{2+} were markedly suppressed or abolished. Addition of exogenous arachidonate increased tissue cAMP and media PGE at both 750 and 1,650 mosM, whereas palmitate and stearate had no effect on cAMP at either osmolality. The actions of Ca^{2+} and arachidonate to increase cAMP and PGE accumulation were abolished by the cyclo-oxygenase inhibitors, indomethacin and meclofenamate. They were also abolished by exclusion of molecular O_2, which serves as cosubstrate with arachidonate in prostaglandin synthesis. At maximally effective concentrations, exogenous PGE_2 and arachidonate produced similar increases in inner medullary cAMP. The maximal effects of the two agents on cAMP were not additive, but were expressed in the absence of Ca^{2+} at both 750 and 1,650 mosM. However, in marked contrast to the O_2-dependent action of arachidonate, PGE_2 increased cAMP in the presence or absence of O_2. Comparison of the separate actions of urea and NaCl indicated that suppression of Ca^{2+}-responsive [^{14}C]arachidonate release, PGE, and cAMP accumulation at 1,650 mosM reflected primarily an effect of urea, whereas hypertonic NaCl, mannitol, and sucrose alone stimulated inner medullary cAMP and PGE accumulation by a pathway which did not require extracellular Ca^{2+}. Analogous to the actions of hypertonic urea, tetracaine and mepacrine inhibited the actions of Ca^{2+} plus A23187 to stimulate [^{14}C]-arachidonate release, PGE, and cAMP accumulation. Inhibition of PGE and cAMP accumulation by tetracaine and mepacrine was also overcome by arachidonate.

The results suggest that high osmolality media with urea as a major solute reduces inner medullary cAMP content, at least in part, through effects on Ca^{2+}-dependent prostaglandin synthesis. Inhibition of PGE synthesis, in turn, may be the result of osmotic suppression of Ca^{2+}-dependent release of arachidonate, the availability of which is often rate limiting to prostaglandin generation.

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INTRODUCTION

The osmolality of the renal inner medulla varies over a wide range under physiologic conditions.

The effects of such fluctuations in tonicity on basal and hormone-responsive metabolic activities of this tissue are not well defined. Previous studies of rat inner medulla have indicated that solute concentration may be an important determinant of basal and arginine vasopressin (AVP)[1]-responsive cyclic AMP (cAMP) accumulation (1). Thus, as media osmolality is increased, basal cAMP content of inner medullary slices declines markedly, whereas AVP-responsive cAMP is either unchanged or enhanced (1). Consistent with the effects on tissue cAMP content, the basal activity of soluble cAMP-dependent protein kinase also falls in inner medulla, and AVP-responsive enzyme activity is concurrently enhanced at higher osmolalities (1, 2). These observations suggest that osmolality influences not only inner medullary content of cAMP, but also the expression of at least one recognized cellular action of cAMP, through which other cellular actions of the nucleotide are thought to be mediated (3).

The mechanisms by which changes in osmolality alter inner medullary cAMP accumulation have not been clarified. Osmotic effects on tissue cAMP levels were not accounted for by fluctuations in tissue wet weight, ATP content, or an altered partitioning of cAMP between tissue and media as a function of media tonicity (1, 2). Earlier observations have indicated that the high cAMP levels encountered in rat inner medullary slices incubated in standard Krebs buffer (305 mosM) and at the high gas-phase O_2 content routinely employed in such in vitro studies is related to an accelerated rate of formation of prostaglandin (PG)E_2, a well-recognized agonist of cAMP in renal medulla (4, 5). Exposure of inner medulla to a high O_2 tension has been shown to enhance PGE_2 synthesis, and thereby tissue cAMP accumulation (4–6). The effects of osmolality on inner medullary prostaglandin synthesis have also been studied previously (6–9). Stimulation (7, 9), inhibition (6), or no effect (8) on this process by high osmolality have been observed, perhaps in part a result of the different solutes employed. In the present study, we examined the possibility that the effects of osmolality on rat inner medullary cAMP metabolism might be related to changes in prostaglandin synthesis. Consistent with the solute content of inner medulla (10, 11), urea was employed as a major component of the high osmolality media.

METHODS

Preparation of tissue. Sprague-Dawley rats weighing 350–400 g (female retired breeders) were obtained from Zivic-Miller Laboratories, Pittsburgh, Pa. Rats were fasted for 18 h before study but allowed ad lib. water. After anesthetization with pentobarbital (5 mg/100 g body wt, i.p.), kidneys were excised and chilled immediately in 0.85% NaCl at 0–4°C. The renal capsules were removed and slices of inner medulla prepared with a Stadie-Riggs microtome as previously described in detail (4).

Slice incubations and determination of cAMP content. Inner medullary slices were incubated in 25-ml flasks with 2 ml of standard Krebs-Ringer bicarbonate buffer (total osmolality; 305 mosM; total Na^+, 145 mM; HCO_3^-, 25 mM; Ca^{2+}, 1.5 mM; Mg^{2+}, 1.2 mM; pH 7.4) containing 5 mM glucose and 1 mg/ml fatty acid-free bovine serum albumin (standard Krebs-Ringer bicarbonate-glucose buffer), or in standard Krebs-Ringer bicarbonate-glucose buffer 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^+ (150 mM NaCl) and 380 mM urea. Buffer with a total osmolality of 1,650 mosM contained 365 mM Na^+ (340 mM NaCl) and 900 mM urea, whereas that with a total osmolality of 2,500 mosM contained 500 mM Na^+ and 1,485 mM urea. Ca^{2+}- and Mg^{2+}-free buffers contained 2 mM EGTA. In these buffers $CaCl_2$ and $MgSO_4$ were replaced by equimolar concentrations of NaCl and Na_2SO_4, respectively. In K^+-free buffer, KCl and KH_2PO_4 were replaced by the corresponding sodium salts. In experiments examining the effects of Ca^{2+}- or Mg^{2+}-free buffer, 2 mM EGTA was also routinely added to the Ca^{2+}- and Mg^{2+}-replete buffer. In complete buffers containing 2 mM EGTA the Ca^{2+} concentration was increased to 3.5 mM and Mg^{2+} to 1.2 mM to maintain a concentration of uncomplexed Ca^{2+} and Mg^{2+} equivalent to that present in complete buffer without EGTA (1.5 mM Ca^{2+} and 1.2 mM Mg^{2+}). 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 verified with a vapor pressure osmometer (model 5100, Wescor Inc., Logan, Utah). Unless otherwise indicated, all stock buffers were gassed with 5% O_2, 5% CO_2 balance N_2 for 10 min and tightly stoppered. As previously reported (1), the differences between pre- and postincubation wet weight of inner medullary slices exposed to the range of osmolality generally employed in the present study (305–1,650 mosM) were small and insignificant (<20%). Accordingly, inner medullary slices were maintained at 0–4°C on filter paper moistened with 0.85% NaCl, blotted, and weighed to the nearest 0.1 mg on a Roller Smith balance immediately before incubation. All results were expressed on the basis of preincubation wet weight. Slice incubations were conducted in a Dubnoff metabolic shaker at 37°C agitated at 100 cycle/min.

Unless otherwise indicated, all slices were preincubated in 1,650 mosM buffer, 5% O_2, 5% CO_2 (balance N_2) for 20 min before transfer to the experimental buffer, a procedure which rapidly stabilized inner medullary cAMP at low levels (1, 2). Incubations for the determination of cAMP content were conducted in the presence or absence of the cyclic nucleotide phosphodiesterase inhibitor, 2 mM 3-isobutyl 1-methyl xanthine (IBMX) as indicated in Results. Routinely, a total of 40–50 mg of inner medulla was present per flask in incubations conducted in the absence of IBMX, whereas 10–15 mg of tissue was used in incubations containing the phosphodiesterase inhibitor. cAMP content of tissue slices was extracted with 50 mM sodium acetate pH 4.0 at 95°C and assayed by the protein binding method (4, 12, 13). Authenticity of the determinations was verified as previously described (4, 12).

Extraction and assay of PGE content. PGE accumulation

[1] *Abbreviations used in this paper*: AVP, arginine vasopressin; cAMP, cyclic AMP; PG, prostaglandin; IBMX, 3-isobutyl 1-methyl xanthine.

in the media was assessed during incubations of inner medullary slices at different osmolalities. [^{3}H]PGE_2 (1,500 cpm) was added to 1.0 ml of media before storage at −20°C. Samples were extracted and assayed for PGE content within 2 wk. The incubation media was adjusted to pH 3–3.5 with 1 N HCl and extracted two times with a total of 15 vol of $CHCl_3$. The $CHCl_3$ extract was evaporated to dryness under N_2 and redissolved in 1 ml of $CHCl_3$. Prostaglandins of the A, E, and F series were isolated by silicic acid chromatography (14) after preliminary separation of prostaglandins from neutral lipids and phospholipids (15, 16). The PGE fraction was evaporated to dryness under N_2 and the residue dissolved in 0.5 ml of 10 mM sodium phosphate, 0.15 M NaCl, pH 7.0 containing 1% (vol/vol) normal rabbit serum. Recovery of PGE, as estimated from that of [^{3}H]PGE_2 was 65–70%. Final values were corrected for recovery. PGE was determined by radioimmunoassay after the general procedures of Stylos et al. (17), utilizing antiserum obtained commercially (Regis Chemical Co., Morton Grove, Ill.). Appropriate dilutions of the samples or PGE_2 standards were incubated for 4 h with antiserum to PGE and [^{3}H]PGE_2 (1,500 cpm) in a total volume of 1 ml of 10 mM sodium phosphate, 0.15 M NaCl buffer, pH 7.0, containing 60 μg/ml of carrier rabbit gamma globulin. Antibodies to PGE were precipitated by incubation for 16–20 h at 4°C with goat anti-rabbit gamma globulin, dissolved in 1 ml of 0.1 N NaOH, and counted by liquid scintillation spectrometry. When plotted as logit-log functions, standard curves were linear from 0.1 to 10 ng PGE_2 per tube. In preliminary experiments tetracaine (0.5 mM), ionophore A23187 (5 μM), meclofenamate (50 μg/ml), indomethacin (100 μg/ml) or their vehicles in aqueous solution did not interfere with the prostaglandin assay. When tissue-free unextracted 1,650 or 750 mosM buffer was tested in the assay, significant displacement of [^{3}H]PGE_2 from the antiserum was observed. However, after the extraction, neither 750 or 1,650 mosM buffer interfered with the immunoassay. With the antibody and assay technique used, cross-reactivity with $PGF_{2\alpha}$, PGA_2, PGD_2, 6-keto $PGF_{1\alpha}$, and arachidonate was ~2.1, 0.7, 1.9, 1.6, and 0.075%, respectively. Chromatographic isolation of the media PGE fraction abolished contamination by either added arachidonate or $PGF_{2\alpha}$, as assessed by the recoveries of labeled arachidonate and $PGF_{2\alpha}$ in the PGE fraction, and by immunoassay of extracted media to which 0.5 mM arachidonate or 0.1 mM $PGF_{2\alpha}$ had been added. Validity of the PGE determinations was indicated by linearity with sample dilution, and the quantitative recovery of PGE_2 added to sample extracts. In addition, release of measurable immunoreactive PGE into the media of inner medullary incubates was reduced to undetectable levels by the presence of indomethacin (see Results). PGE content of the media is expressed as nanograms per milligram of tissue wet weight. Routinely, PGE accumulation in the media and cAMP accumulation in the tissue was monitored concurrently using samples from the same incubates.

Determination of the rate of accumulation of ^{14}C in media from inner medullary slices prelabeled with [^{14}C]-arachidonic acid. Approximately 200 mg of inner medullary slices were incubated at 37°C for 20 min in 2 ml of Ca^{2+}-, Mg^{2+}-, and albumin-free 1,650 mosM buffer containing 0.5 μCi (4.5 μM) of [^{14}C]arachidonic acid, 2 mM EGTA, and routinely 5 μM ionophore A23187. At the end of the 20-min prelabeling period the slices were washed briefly at 37°C in fresh Ca^{2+}- and Mg^{2+}-free 1,650 mosM buffer to which had been added 15 mg/ml fatty acid-free bovine serum albumin. Slices were then incubated at 37°C for 25 min in Ca^{2+}- and Mg^{2+}-free 750 or 1,650 mosM buffer containing 15 mg/ml albumin. Slices were then transferred to fresh buffer of the same osmolality and composition for a final incubation period during which Ca^{2+} or Mg^{2+} were added. At 5-min intervals during this final incubation, a 50-μl aliquot of the incubation media was removed and counted. Where indicated in the results, slices were prelabeled by initial incubation with 0.5 μCi of [^{14}C]palmitate rather than arachidonate. All media ^{14}C-counting rates were at least twice background. In some experiments, slices were extracted in 20 vol of $CHCl_3$/CH_3OH, 2/1 for lipid analysis immediately before the addition of Ca^{2+}. Approximately 7% of added ^{14}C was present per 200 mg inner medulla just before addition of Ca^{2+} (150,000 dpm/g wet wt) in slices initially incubated with either [^{14}C]-arachidonate or palmitate. Of the total tissue-associated ^{14}C, 90% or more was recovered in the lipid fraction (see below). In 750 mosM buffer, ~20% of total ^{14}C was recovered in the media 25 min after Ca^{2+} addition (calculated as media ^{14}C/media ^{14}C plus tissue ^{14}C). The rate of accumulation of ^{14}C was calculated from the counting rates during the 10-min periods immediately before (5–15 min of the final incubation) and after Ca^{2+} addition (15–25 min), and expressed as disintegrations per minute per gram of tissue per minute.

Lipid extraction and analysis. Media or tissue samples derived from duplicate flasks representing the same condition in an experiment were pooled for the purposes of lipid extraction and analysis. Butylated hydroxytoluene (0.25 mM) was added to stored samples. Tissue lipids were extracted with $CHCl_3$/CH_3OH by the general method of Folch et al. (18).

Media obtained was centrifuged immediately after incubation with loss of <10% of radioactivity under all slice incubation conditions. Media lipids were extracted by a technique (16) similar to that described by Bills et al. (15).

Lipids were separated into major classes by silicic acid chromatography (15, 16). Extracts were dissolved in 1 ml $CHCl_3$ and applied to silicic acid columns prepared in $CHCl_3$. Neutral lipids were eluted with 5 ml $CHCl_3$, prostaglandins with 5 ml $CHCl_3$/CH_3OH (19/1), and phospholipids with 6 ml of CH_3OH followed by 4 ml CH_3OH/H_2O (99/1). The eluted fractions were evaporated to dryness under N_2 and redissolved in $CHCl_3$ (neutral lipids and prostaglandins) or $CHCl_3$/CH_3OH (9/1) for phospholipids. The distribution of ^{14}C among fatty acid, triglyceride, or cholesterol ester was determined by chromatography in hexane/ethyl ether/acetic acid (80/20/2) on plates of silica gel G (Redi-Coat G) which had been activated overnight at 70°C. Lipids were located by use of iodine vapor and individual spots transferred to counting vials. All experimental ^{14}C-counting rates were at least twice background.

Recovery of sample radioactivity was monitored at each step during lipid extraction and analysis. Recoveries during extraction of tissue lipids were based on radioactivity in the $CHCl_3$/CH_3OH fraction after passage through glass wool and varied between 80 and 82%. Recoveries during lipid extraction of the media were based on radioactivity present in the clear supernate after centrifugation of the media and varied between 87 and 92%. In addition, samples similar in composition to the incubation media to which [^{14}C]arachidonic acid and [^{3}H]PGE_2 had been added was subjected to the extraction and lipid separation procedures. Recovery in these lipid standards ranged from 91 to 96%.

Statistical significance of differences between mean values are assessed by Student's t test for unpaired values (19). Values shown represent mean±SE of determinations derived from three to four separate experiments. In studies of cAMP and PGE, each condition in a given experiment was represented in triplicate incubation flasks, whereas in studies of ^{14}C release each condition was represented in duplicate incubation flasks. For purposes of statistical analysis the

average value derived from replicate sets of flasks from the same experiment was entered as a single value (df = 4–6, comparing values for any two experimental conditions derived from three to four separate experiments by *t* test for unpaired values).

Materials. Urea, synthetic AVP (grade VI, 367 IU/mg), tetracaine, fatty acids, mannitol, and sucrose were obtained from Sigma Chemical Co., St. Louis, Mo. Carrier rabbit gamma globulin and goat anti-rabbit gamma globulin, P-4 strength, were purchased from Antibodies Inc., Davis, Calif. [^{3}H]PGE_2 was obtained from New England Nuclear, Boston, Mass., and [^{14}C]arachidonic acid from Amersham-Searle, Chicago, Ill. Mepacrine was obtained from ICN K & K Laboratories Inc., Plainview, N. Y. Silicic acid (Unisil) was obtained from Clarkson Chemical Co., Williamsport, Pa., and Redi Coat G, thin-layer plates from Supelco, Inc., Bellefonte, Pa. PGE_2 and PGI_2 were gifts of The Upjohn Company, Kalamazoo, Mich. Sodium meclofenamate and ionophore A23187 were provided by Parke, Davis & Company, Detroit, Mich.; and Eli Lilly and Company, Indianapolis, Ind., respectively. Sources of all other chemicals have been previously described (1, 2, 4).

RESULTS

As shown in Table I, cAMP content of inner medullary slices varied inversely as a function of media osmolality over a range of 305 mosM (standard Krebs bicarbonate buffer) to 1,650 mosM buffer, when osmolality was increased by the addition of urea plus NaCl to the standard buffer. cAMP of slices incubated in 2,500 mosM buffer did not differ significantly from those exposed to 1,650 mosM. Consistent with earlier observations (1), effects of osmolality on inner medullary cAMP were evident at gas-phase O_2 contents of either 5 or 95%. Because the former gas-phase O_2 more closely mimicks the low O_2 tension found in vivo in renal papilla (20, 21), it was employed routinely in the present studies. As also shown in Table I, effects of osmolality on inner medullary cAMP were evident in the presence or absence of the cyclic nucleotide phosphodiesterase inhibitor IBMX, and thus may reflect alterations in cAMP synthesis.

Fig. 1 shows the time-course of changes in inner

TABLE I

Effects of Media Osmolality and Gas-Phase O_2 Content on Inner Medullary cAMP Accumulation in the Presence and Absence of IBMX

Gas-phase O_2	Media osmolality	−IBMX	+IBMX
	mosM	*pmol cAMP/mg tissue*	
5%	305	1.73±0.34	8.90±1.04
	750	0.82±0.19*	5.16±0.73*
	1,650	0.24±0.04*‡	1.85±0.38*‡
	2,500	0.21±0.04*‡	1.57±0.26*‡
95%	305	6.58±0.81	24.8±3.1
	750	4.16±0.67*	15.4±2.1*
	1,650	0.75±0.10*‡	4.64±0.71*‡
	2,500	0.62±0.09*‡	3.96±0.73*‡

Inner medullary slices were preincubated for 20 min at 37°C in 1,650 mosM buffer with a gas phase of 5% O_2, 5% CO_2, and 90% N_2. Slices were then transferred to fresh buffers whose total osmolality is indicated in table. The second incubation was continued for an additional 30 min, and where shown, contained 2 mM IBMX. The gas phase contained either 5% or 95% O_2. The Na^+ and urea content of the media with the total osmolalities shown in the table are: 305 mosM, 145 mM Na^+, no urea; 750 mosM, 175 mM Na^+, 380 mM urea; 1,650 mosM, 365 mM Na^+, 900 mM urea; 2,500 mosM, 500 mM Na^+, 1,485 mM urea. Values represent mean±SE of determinations pooled from three separate experiments.

* $P < 0.01$ compared to corresponding value at 305 mosM.

‡ $P < 0.01$ compared to corresponding value at 750 mosM.

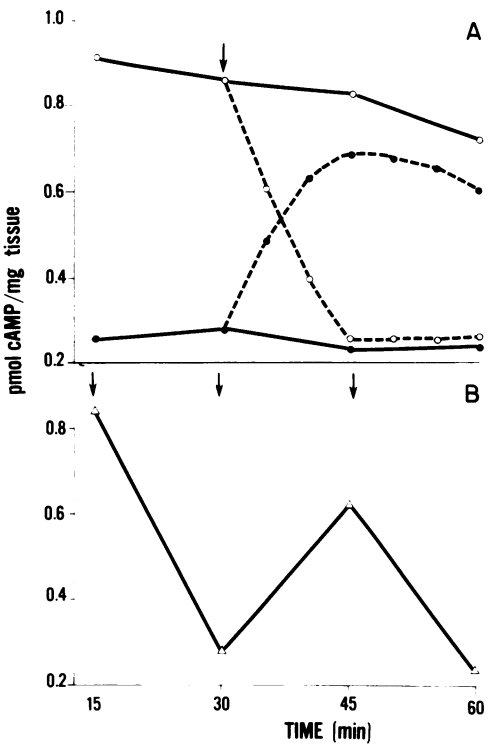

FIGURE 1 Sequential effects of media osmolality on inner medullary cAMP. (A) Inner medullary slices were initially incubated for 30 min at 37°C in either 750 (○ —— ○) or 1,650 (● —— ●) mosM buffer. Some slices were transferred at 30 min, as indicated by the arrow, from either 750 to 1,650 (○ - - - ○) or from 1,650 to 750 (● - - - ●) mosM buffer. Other slices were transferred at 30 min to fresh buffer of the same osmolality. The incubations were then continued for the additional time intervals shown. (B) All inner medullary slices were initially incubated for 15 min at 37°C in 750 mosM buffer. Slices were transferred sequentially at 15-min intervals from 750 to 1,650 mosM (t = 15), from 1,650 to 750 mosM (t = 30), and finally from 750 to 1,650 mosM (t = 45), as indicated by the arrows. Incubations were continued for 15 min after transfer of slices to a buffer of different osmolality. Three of the transferred slices were extracted for cAMP content 15 min after each transfer.

medullary cAMP content produced by transfer of slices to a buffer of different osmolality. As shown in Fig. 1A, cAMP of slices exposed to 1,650 mosM buffer for an initial 30-min period and then transferred to 750 mosM buffer, rose approximately threefold during the 15-min period after transfer. Conversely, cAMP of slices initially incubated for 30 min at 750 mosM and then transferred to 1,650 mosM media fell markedly over 15 min. cAMP content of slices initially incubated for 15 min in 750 mosM media and then transferred sequentially to 1,650, 750, and 1,650 mosM media at 15-min intervals varied inversely over this time period as a function of osmolality of the incubation media (Fig. 1B).

Table II shows alterations in cAMP in inner medullary slices incubated in complete 750 or 1,650 mosM buffer vs. those incubated in buffers of corresponding osmolalities but devoid of Ca^{2+} or Mg^{2+}. All buffers contained 2 mM EGTA. cAMP content of slices incubated in Ca^{2+}- and Mg^{2+}-free 750 mosM buffer were significantly lower than cAMP levels of slices exposed to identical osmotic conditions in complete buffer. At 1,650 mosM, no effect of Ca^{2+} or Mg^{2+} exclusion was evident. Addition of 3.5 mM Ca^{2+} to Ca^{2+}- and Mg^{2+}-deprived inner medulla incubated at 750 mosM buffer with 2 mM EGTA increased cAMP two- to threefold, but did not influence cAMP of slices incubated in 1,650 mosM buffer. Although cAMP of slices incubated in Ca^{2+}-deficient 750 mosM buffer was clearly lower than that observed in complete 750 mosM buffer, they remained significantly above cAMP levels observed in either complete or Ca^{2+}-deficient 1,650 mosM buffer. The effects of Ca^{2+} deprivation to reduce cAMP and of the subsequent Ca^{2+} addition to increase nucleotide levels were also observed in the presence of IBMX (Table II), and thus may reflect Ca^{2+}-dependent changes in cAMP synthesis. At 750 mosM, addition of 3.5 mM Ca^{2+} detectably increased cAMP within 2 min with peak increases observed by 15 min. No further increases were observed with 6 mM Ca^{2+}. Addition of 1.2 mM Mg^{2+} to inner medullary incubates previously deprived of Ca^{2+} and Mg^{2+} did not detectably alter cAMP at either osmolality. cAMP values of slices incubated in complete 750 or 1,650 mosM buffer without EGTA were not different from those of slices incubated in buffer of the corresponding osmolality but with 2 mM EGTA plus 3.5 mM Ca^{2+} and 1.2 mM Mg^{2+}. Moreover, exclusion of Mg^{2+} alone in buffer containing 3.5 mM Ca^{2+} plus 2 mM EGTA did not alter cAMP compared to values seen in complete 750 or 1,650 mosM buffer. K^+-free buffer was also without effect on inner medullary cAMP at either osmolality (not shown). Thus, the effects of Ca^{2+} deprivation to prevent the increase in cAMP observed upon transfer of slices from 1,650 to 750 mosM buffer were selective relative to extracellular Mg^{2+} or K^+ deprivation and were specifically reversed by addition of Ca^{2+}. It should be emphasized that preincubation of slices with EGTA was required to demonstrate the reduction in cAMP in Ca^{2+}-free buffer. Prior ex-

TABLE II

Effects of Exclusion of Extracellular Ca^{2+} and Mg^{2+} on Basal and AVP-Stimulated cAMP Accumulation in Inner Medullary Slices Incubated at 750 vs. 1,650 mosM

	Final additions					
	−IBMX				+IBMX	
Incubation conditions	None	Mg^{2+}	Ca^{2+}	AVP	None	Ca^{2+}
	pmol cAMP/mg tissue					
750 mosM						
Complete buffer	1.21±0.22	—	1.34±0.19	2.26±0.31§	6.42±0.83	6.60±0.91
Ca^{2+}- and Mg^{2+}-free	0.45±0.04*	0.39±0.04	1.12±0.16§	2.53±0.33§	2.96±0.44*	6.74±0.79§
1,650 mosM						
Complete buffer	0.31±0.04‡	—	0.33±0.07‡	2.41±0.26§	2.05±0.36‡	1.93±0.42‡
Ca^{2+}- and Mg^{2+}-free	0.26±0.03‡	0.32±0.04	0.30±0.04‡	1.98±0.31§	1.78±0.34‡	2.17±0.41‡

All inner medullary slices were preincubated for 20 min at 37°C in 1,650 mosM buffer with a gas phase of 5% O_2, 5% CO_2, and 90% N_2. Slices were then transferred to 750 or 1,650 mosM buffer (5% O_2) with and without 2 mM IBMX and the incubation continued for an additional 40 min. All buffers contained 2 mM EGTA. Complete buffer contained 1.2 mM $MgSO_4$ and 3.5 mM $CaCl_2$ to give a final calculated Ca^{2+} concentration of 1.5 mM in excess of EGTA. Mg^{2+} (1.2 mM) or Ca^{2+} (3.5 mM) was added to the incubates at 20 min during the second incubation, where indicated. AVP was added to some slice incubations for the final 2 min at a concentration of 30 nM. Values represent mean±SE from three (with IBMX) to four (without IBMX) separate experiments.

* $P < 0.01$ compared to corresponding value in complete buffer of the same osmolality.

‡ $P < 0.01$ compared to the value in 750 mosM buffer with the corresponding Ca^{2+} and Mg^{2+} content.

§ $P < 0.01$ compared to corresponding value in the absence of a final addition.

clusion of Ca^{2+} alone was inadequate. Absolute cAMP responses to a maximal stimulatory concentration of AVP (30 nM) were comparable under the various incubation conditions examined in Table II. However, in Ca^{2+}-deprived slices incubated at 750 mosM, proportional increases in cAMP over the corresponding basal (5.6-fold) greatly exceeded those observed in slices incubated in complete buffer of the same osmolality (1.8-fold), and resembled the AVP response observed at 1,650 mosM (7- to 8-fold over basal).

Table III shows the effects of addition of Ca^{2+} or Mg^{2+} on tissue cAMP and on concurrent accumulation of immunoreactive PGE in the media in inner medullary incubates initially deprived of Ca^{2+} and Mg^{2+}. As shown in Table III, in slices incubated in Ca^{2+}- and Mg^{2+}-free 750 mosM buffer, addition of Ca^{2+} significantly increased both tissue cAMP content and accumulation of PGE in the media. Mg^{2+} was without effect on either parameter. At 750 mosM, the presence of the divalent cation ionophore A23187, enhanced the PGE as well as the cAMP response to Ca^{2+}, whereas Mg^{2+} remained ineffective in the presence of the ionophore. At 1,650 mosM, Ca^{2+} did not detectably alter tissue cAMP or media accumulation of PGE in the presence or absence of A23187 (Table III). The failure of Ca^{2+} to increase accumulation of PGE in the media at 1650 mosM was not attributable to retention of generated PGE by the tissue at this osmolality. PGE was found predominantly in the media at 1,650 mosM, with little or no assayable PGE recovered in the slice extracts. This is consistent with findings in inner medulla at lower osmolality (6, 22, 23) and with the rapid release of newly generated prostaglandins observed in other tissues (24).

To assess the relationship between Ca^{2+}-dependent changes in inner medullary cAMP content and prostaglandin synthesis observed as a function of osmolality, the effects on cAMP of the cyclooxygenase inhibitors, indomethacin and meclofenamate, and of O_2 deprivation were examined. cAMP of oxygenated inner medullary slices incubated in complete 750 mosM buffer without indomethacin was significantly higher than that of slices incubated in the presence of indomethacin or in the absence of O_2 (750 mosM, 5% O_2, 8.21±1.23; plus 100 μg/ml indomethacin, 2.95±0.37; no O_2, 2.69±0.37 pmol cAMP/mg tissue). By contrast, addition of indomethacin or exclusion of O_2 had little influence on cAMP levels of inner medulla incubated in 1,650 mosM buffer (1,650 mosM, 5% O_2; 2.25±0.38; plus indomethacin, 1.93±0.27; no O_2, 1.72±0.23). Moreover, as shown in Table IV, cAMP of slices incubated in the absence of Ca^{2+} at 750 mosM was lower than that of slices incubated in complete buffer. By contrast, exclusion of Ca^{2+} had little effect on cAMP at 1,650 mosM. Indomethacin and meclofenamate blocked the increase in inner medullary cAMP seen when Ca^{2+} was added to Ca^{2+}-free 750 mosM buffer. Similarly, O_2 exclusion also

TABLE III

Effects of Ca^{2+} on the Accumulation of cAMP in the Tissue and PGE in the Medium of Inner Medullary Slice Incubates Exposed to 750 vs. 1,650 mosM

Incubation condition	Final additions					
	cAMP			PGE		
	None	Mg^{2+}	Ca^{2+}	None	Mg^{2+}	Ca^{2+}
	pmol/mg tissue			*ng/mg tissue*		
−A23187						
750 mosM	0.46±0.04	0.54±0.05	1.25±0.11*	4.63±0.71	5.27±0.84	7.22±1.13*
1,650 mosM	0.27±0.03‡	0.31±0.04‡	0.35±0.04‡	3.92±0.57	0.44±0.61	4.35±0.62‡
+A23187						
750 mosM	0.33±0.04§	0.39±0.05	2.17±0.32§	4.24±0.57	4.72±0.53	12.9±1.5§
1,650 mosM	0.29±0.03	0.32±0.04	0.44±0.06‡	4.67±0.59	5.12±0.62	4.54±0.64‡

All inner medullary slices were preincubated for 20 min at 37°C in Ca^{2+}- and Mg^{2+}-free 1,650 mosM buffer, in the presence and absence of 5 μM ionophore A23187. A23187 was added as a solution in ethanol. Flasks which did not receive A23187 received an addition of ethanol (0.25%, final concentration). Slices were then transferred to Ca^{2+}- and Mg^{2+}-free 750 or 1,650 mosM with or without the ionophore and the incubation continued for an additional 40 min. The gas phase was 5% O_2, 5% CO_2, 90% N_2 during the entire period of study, and all buffers contained 2 mM EGTA. Where indicated, 3.5 mM Ca^{2+} or 1.2 mM Mg^{2+} was present during the final 20 min of the second incubation. At the end of the incubation, slices were extracted and assayed for cAMP content, and the final incubation medium was frozen at −20°C for extraction and assay of PGE content. Values represent mean±SE of determinations from four separate experiments.

* $P < 0.01$ compared to corresponding value in the absence of Ca^{2+} and Mg^{2+}.

‡ $P < 0.01$ compared to corresponding value at 750 mosM.

§ $P < 0.01$ compared to corresponding value without ionophore A23187.

TABLE IV

Effects of Indomethacin, Meclofenamate, and O_2 Exclusion on Ca^{2+}-, Arachidonate-, and PGE_2-Induced cAMP Accumulation in Inner Medullary Slices

	−A23187				+A23187	
	None	Ca^{2+}	Arachidonate	PGE_2	None	Ca^{2+}
	pmol cAMP/mg tissue					
750 mosM						
Complete buffer	8.21±1.23	7.25±1.18	14.3±1.3*	17.8±2.4*	6.89±0.94	9.87±1.73
Ca^{2+} and Mg^{2+} free	2.53±0.31‡	5.86±1.16*	14.3±1.9*	14.2±1.9*	1.67±0.24‡	9.16±1.32*
+ Indomethacin	1.47±0.22§	1.55±0.16§	1.82±0.19§	14.8±1.9*	1.68±0.31	1.71±0.28§
+ Meclofenamate	1.69±0.28§	2.07±0.38§	1.43±0.27§	15.2±2.4*	1.75±0.25	2.10±0.34§
No O_2	1.82±0.29§	2.12±0.34§	1.95±0.25§	13.9±1.8*	1.59±0.29	1.73±0.35§
1,650 mosM						
Complete buffer	2.25±0.38‖	2.07±0.29‖	12.2±2.2*	13.1±1.8*	2.13±0.37‖	2.04±0.26§‖
Ca^{2+} and Mg^{2+} free	1.43±0.24‖	1.65±0.27‖	12.2±2.2*	10.9±2.4*	1.41±0.24	1.93±0.31‖

All inner medullary slices were preincubated for20 min at 37°C in 1,650 mosM buffer (5% O_2). Slices were then transferred to 750 or 1,650 mosM buffer containing 2 mM IBMX. 100 μg/ml indomethacin or 50 μg/ml meclofenamate was added to the second 40-min incubation where shown. Gas phase for the second incubation consisted of 5% O_2, 5% CO_2, 90% N_2, or 95% N_2, 5% CO_2 as indicated. All buffers contained 2 mM EGTA. Complete buffer contained 3.5 mM $CaCl_2$ and 1.2 mM $MgSO_4$. Ca^{2+} and Mg^{2+} were excluded whereas ionophore A23187 (5 μM) was present during both incubations when stated. Where indicated, 0.1 mM arachidonate or 3.5 mM $CaCl_2$ was present for the final 20 min, whereas 0.1 mM PGE_2 was added for the final 5 min of incubation. Arachidonate was added as the Na^+ salt complexed to fatty acid-free albumin. 1 mg/ml albumin was added to those flasks which did not receive an addition of arachidonate. Values represent the mean±SE of determinations from three separate experiments.

* $P < 0.01$ compared to corresponding value in the absence of a final addition.

‡ $P < 0.01$ comparing value in Ca^{2+}- and Mg^{2+}-free buffer to value in complete buffer of the same osmolality.

§ $P < 0.01$ compared to value obtained in Ca^{2+}- and Mg^{2+}-free 750 mosM buffer at 5% O_2 in the absence of indomethacin and meclofenamate.

‖ $P < 0.01$ compared to corresponding value at 750 mosM.

abolished the action of Ca^{2+} to increase inner medullary cAMP. The inhibitory effects of indomethacin, meclofenamate, or O_2 exclusion on this Ca^{2+} action were expressed in both the presence and absence of A23187 (Table IV). As also shown in this table, the addition of 0.1 mM arachidonate to Ca^{2+}-deprived inner medulla markedly increased cAMP levels. However, unlike the effects of Ca^{2+}, the action of arachidonate to increase cAMP was expressed in both 750 and 1,650 mosM buffer. In 750 mosM buffer, the actions of arachidonate to increase inner medullary cAMP were abolished by indomethacin, meclofenamate, and by O_2 deprivation. In other experiments (not shown) cAMP responses were detectable (50% to twofold increases over the corresponding basal value) with 0.025 mM arachidonate in both buffers and appeared maximal at 0.1 mM. No further increase in cAMP was observed in slices exposed to 0.5 mM arachidonate. Absolute cAMP accumulation in response to arachidonate was somewhat greater in slices incubated at the lower osmolality. Maximal arachidonate increased cAMP within 2 min at both osmolalities with peak responses evident by 15 min. By contrast, 0.5 mM palmitate, stearate, or oleate were without detectable effect on inner medullary cAMP at either osmolality.

Analogous to the findings with arachidonate, PGE_2 markedly increased the cAMP levels of oxygenated inner medullary slices incubated in complete 750 or 1,650 mosM buffer, and was effective in the absence of Ca^{2+} (Table IV). However, while the action of arachidonate to increase inner medullary cAMP at either osmolality was markedly suppressed or totally abolished by addition of indomethacin or by exclusion of O_2, the absolute cAMP accumulation in response to PGE_2 was not significantly changed by addition of indomethacin or by exclusion of O_2. Because of the reduction in basal cAMP induced by incubation of slices at 1,650 mosM, the addition of indomethacin or the exclusion of O_2 or Ca^{2+} in slices incubated in 750 mosM buffer, relative cAMP responses to PGE_2 were enhanced under all the latter conditions. The concentrations of arachidonate and PGE_2 employed were maximal with respect to stimulation of cAMP accumulation. In oxygenated slices, the combined addition of 0.1 mM PGE_2 and arachidonate gave cAMP responses not different from those seen with either agent alone (Table IV), a finding consistent

with stimulation of a common pool of inner medullary cAMP by both agents. cAMP responses to PGI_2 under the incubation conditions shown in Table IV were comparable to those observed in response to PGE_2.

Table V shows the action of Ca^{2+} on accumulation of [^{14}C]arachidonate and PGE in the media of inner medullary incubates at 750 or 1,650 mosM. All slices were initially incubated in Ca^{2+}- and Mg^{2+}-free buffers with A23187. Under these conditions, a final addition of Ca^{2+} increased the rate of ^{14}C accumulation in the media five- to sixfold in slices prelabeled with [^{14}C]arachidonate and incubated at a final osmolality of 750 mosM. In slices incubated at a final osmolality of 1,650, addition of Ca^{2+} resulted in only a 50% increase in the rate of ^{14}C accumulation. Addition of Mg^{2+} did not stimulate ^{14}C accumulation at either osmolality. When A23187 was omitted, Ca^{2+} addition increased ^{14}C accumulation 0.6- to 2.1-fold over basal at 750 mosM and was without detectable effect at 1,650 mosM. As shown in Fig. 2, the accelerated rate of ^{14}C accumulation in the media induced by Ca^{2+} (+A23187) was evident for ~15 min in 750 mosM buffer, and then returned to basal levels.

Chromatographic analysis of lipid extracts of media sampled after Ca^{2+} addition indicated that the accumulated ^{14}C was present predominantly as free fatty acid (83±11% at 750 mosM vs. 80±10% at 1,650 mosM, $n = 4$). Most of the residual ^{14}C in the media was present as prostaglandin (6±2% at 750 mosM vs. 3±1% at 1,650 mosM) and phospholipid (11±3% at 750 mosM vs. 17±7% at 1,650 mosM). The differences in the percentage distribution of media ^{14}C among the lipid fractions observed as a function of osmolality were not statistically significant. When [^{14}C]palmitate rather than [^{14}C]arachidonate was employed to prelabel inner medullary slices as described in Table V, no effect of Ca^{2+} to accelerate the rate of ^{14}C accumulation in the media was observed at either 750 or 1,650 mosM. Failure of Ca^{2+} to accelerate the rate of ^{14}C accumulation in the media in slices initially incubated with [^{14}C]palmitate was not attributable to a lack of labeling of the tissue lipid pool. Approximately 5% of initially added ^{14}C (0.5 μCi of [^{14}C]palmitate) was found in the slice lipid fraction before Ca^{2+} addition, a value not different from that observed in tissue prelabeled with [^{14}C]arachidonate.

Total ^{14}C was not detectably different in lipid extracts of slices prelabeled with [^{14}C]arachidonate and then

TABLE V

Effects of Ca^{2+} on Accumulation of [^{14}C]Arachidonate and PGE in Media of Inner Medullary Incubates at 750 vs. 1,650 mosM

	Final additions				
	^{14}C Accumulation rate		PGE		
Incubation conditions	None	Ca^{2+}	None	Ca^{2+}	Arachidonate
	dpm/min/g tissue		*ng/mg tissue*		
750 mosM (5% O_2)	423±78	2,347±373*	4.31±0.56	12.4±2.1*	28.1±4.4*
+ Indomethacin	418±76	2,161±305*	ND	ND	ND
− O_2	581±84	2,672±387	ND	ND	ND
1,650 mosM (5% O_2)	456±62	708±83‡	3.34±0.51	3.71±0.52‡	21.6±3.8*
+ Indomethacin	489±56	697±92‡	ND	ND	ND

Inner medullary slices (60–80 mg for PGE and 200 mg for ^{14}C accumulation rate) were preincubated for 20 min at 37°C in Ca^{2+}- and Mg^{2+}-free 1,650 mosM buffer, containing 5 μM ionophore A23187 and 2 mM EGTA. The gas phase contained 5% O_2. Incubations for the determination of ^{14}C accumulation rate contained 0.5 μCi of [^{14}C]arachidonate (4.5 μM). At the end of the first 20-min incubation the slices were washed in 1,650 mosM Ca^{2+}- and Mg^{2+}-free buffer and transferred to 750 or 1,650 mosM Ca^{2+}- and Mg^{2+}-free buffer containing A23187 for a second 25-min incubation. With the exception of the initial 20-min prelabeling period 15 mg/ml of fatty acid-free albumin was present in all incubation media used for the determination of ^{14}C accumulation, but not for PGE accumulation. Slices were transferred to fresh buffer for a final 35 min under conditions identical to those employed during the second incubation period. Where shown, Ca^{2+} or arachidonate was added to the slice incubation for the final 20 min at concentrations of 3.5 and 0.1 mM, respectively. The rate of accumulation of ^{14}C by slices prelabeled with [^{14}C]arachidonate was calculated from the 10-min periods immediately before and after addition of Ca^{2+}. Values represent the mean±SE of determinations from four experiments.

* $P < 0.01$ compared to corresponding value without Ca^{2+} or arachidonate addition.

‡ $P < 0.01$ compared to corresponding value at 750 mosM. ND, not detectable.

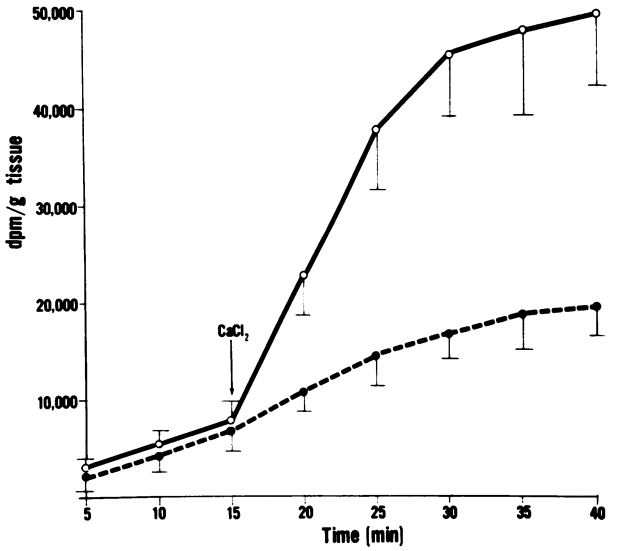

FIGURE 2 Time-course of the actions of Ca^{2+} on the accumulation of [^{14}C]arachidonate in media of inner medullary incubates. Inner medullary slices were preincubated for 20 min at 37°C in Ca^{2+}- and Mg^{2+}-free 1,650 mosM buffer containing 5 μM ionophore A23187 and 0.5 μCi of [^{14}C]arachidonate. Slices were then washed in Ca^{2+}- and Mg^{2+}-free 1,650 mosM buffer containing 15 mg/ml albumin and transferred to 750 mosM (○) or 1,650 (●) buffer with albumin for an additional 25 min of incubation. Slices were then transferred to fresh Ca^{2+}- and Mg^{2+}-free 750 mosM (○) or 1,650 mosM (●) buffer of identical osmolality and composition and the incubation continued for an additional 40 min. The times referred to in the figure are those after the start of the final 40-min incubation. Ca^{2+} was then added to some slice incubates (t = 15) to give a final concentration of 3.5 mM and the incubation continued to t = 40. All buffers contained 2 mM EGTA with a gas phase of 5% O_2, 5% CO_2, and 90% N_2 throughout. Values shown represent mean±SE of media ^{14}C from four separate experiments.

exposed to either 750 or 1,650 mosM buffer before the addition of Ca^{2+}. Under both conditions of incubation ~5% of initially added ^{14}C (0.5 μCi) was found in the lipid fraction of slices harvested before Ca^{2+} addition during the third incubation (Table V). In slices incubated at 750 mosM, the distribution of ^{14}C in the lipid extract of tissue before addition of Ca^{2+}, revealed 37±6% of label in the neutral lipid fraction, 62±6% as phospholipid, and 0–0.8% as prostaglandin. Corresponding values in slices exposed to 1,650 mosM buffer before Ca^{2+} addition were 35±5% as neutral lipid, 64±5% as phospholipid, and 0–0.6% as prostaglandin. Approximately 75% of the ^{14}C in the tissue neutral lipid fraction was present as triglyceride and cholesterol ester, with the residual as free fatty acid. As also shown in Table V, indomethacin or O_2 deprivation did not alter Ca^{2+} action on the accumulation of [^{14}C]arachidonate in the media. By contrast, detectable accumulation of immunoreactive PGE in 750 mosM buffer was abolished by indomethacin or O_2 deprivation, in either the presence or absence of Ca^{2+} (Table V). Addition of 0.1 mM exogenous arachidonate markedly increased PGE release from inner medulla in Ca^{2+}-free 750 or 1,650 mosM buffer. PGE accumulation was not detectable in the presence of indomethacin or during O_2 exclusion (Table V). Changes in tissue cAMP in response to Ca^{2+} and arachidonate at 750 and 1,650 mosM in these experiments paralleled changes in media PGE. Thus, analogous to results shown in Table IV, addition of arachidonate, but not Ca^{2+}, to the final incubation media significantly increased cAMP at 1,650 mosM, whereas both agents were effective at 750 mosM.

Several observations suggested that the Ca^{2+}-induced acceleration of accumulation of [^{14}C]arachidonate in the media at 750 mosM reflected primarily enhanced tissue release of ^{14}C rather than a diminished rate of label uptake. Thus, addition of excess (0.5 mM) unlabeled arachidonate immediately after Ca^{2+} addition during the third (final) incubation period of the prelabeling experiments did not prevent the acceleration of [^{14}C]arachidonate accumulation shown in Fig. 2. Moreover, in separate studies, slices were incubated under conditions identical to those described in Table V for the prelabeling experiments except that the addition of the [^{14}C]arachidonate was omitted during the initial incubation period. During the third incubation period, sufficient [^{14}C]arachidonate was added to the incubate immediately after the addition of Ca^{2+} to yield a media concentration of 4,000 dpm/ml. This approximated the concentration of ^{14}C in the media in the prelabeling studies at the time of Ca^{2+} addition. Disappearance of ^{14}C from the media over the subsequent 10-min period was negligible and not different at either 750 or 1,650 mosM. Only 3% of added ^{14}C was associated with the tissue at the end of this 10-min period. The slow rate of tissue uptake of [^{14}C]arachidonate under conditions of the final incubation may be related to the presence of albumin (15 mg/ml), as suggested by previous studies in platelets (15). Albumin was omitted during the first incubation, where [^{14}C]-arachidonate was added in the prelabeling studies (Table V). Accordingly, it seems likely that Ca^{2+}-induced acceleration of [^{14}C]arachidonate accumulation in media at 750 mosM reflects predominantly an acceleration of release from the tissue.

Table VI shows the effects of tetracaine and mepacrine on Ca^{2+}-responsive cAMP, [^{14}C]arachidonate, and PGE in inner medulla. These studies were conducted in the presence of 5 μM A23187. Tetracaine (0.5 mM) abolished, whereas 0.1 mM mepacrine suppressed, the actions of Ca^{2+} to increase tissue cAMP and media PGE accumulation at 750 mosM. Tetracaine and mepacrine also suppressed Ca^{2+} effects on media accumulation of ^{14}C in slices prelabeled with [^{14}C]-arachidonate and incubated under the same conditions. The actions of arachidonate to increase cAMP and

TABLE VI

Effects of Tetracaine and Mepacrine on Ca^{2+}-Responsive cAMP and the Accumulation of [^{14}C]Arachidonate and PGE in Inner Medullary Incubates

Incubation conditions	Final additions		
	None	Ca^{2+}	Arachidonate
cAMP, *pmol/mg wt*			
750 mosM	0.41±0.06	2.12±0.30‡	2.69±0.48‡
+ Tetracaine	0.37±0.05	0.45±0.05*	2.84±0.37‡
+ Mepacrine	0.48±0.06	1.26±0.21*‡	3.19±0.44‡
^{14}C Accumulation rate, *dpm/min/g tissue*			
750 mosM	386±62	2,279±410‡	—
+ Tetracaine	436±71	683±65*	—
+ Mepacrine	479±75	1,084±235*‡	—
PGE, *ng/mg tissue*			
750 mosM	3.62±0.54	11.7±1.7‡	19.1±2.4‡
+ Tetracaine	3.14±0.46	3.72±0.41*	22.3±2.8‡
+ Mepacrine	2.87±0.53	6.93±0.82*‡	17.4±2.1‡

Experiments were conducted as described in Table VI, except that all slices were finally incubated at 750 mosM. Where indicated, tetracaine was present during the final 35-min incubation; 3.5 mM Ca^{2+} or 0.1 mM arachidonate was added to appropriate flasks for the final 20 min of this third incubation. Values represent mean±SE of determinations from four experiments.

* $P < 0.01$ compared to corresponding value in the absence of tetracaine or mepacrine.

‡ $P < 0.01$ compared to corresponding value in the absence of Ca^{2+} or arachidonate as a final addition.

media PGE accumulation were not inhibited by tetracaine or mepacrine (Table VI).

Table VII shows the effects of urea alone on Ca^{2+}-responsive accumulation of cAMP in tissue and on accumulation of [^{14}C]arachidonate and PGE in media of inner medullary incubates. Ca^{2+} clearly increased tissue cAMP, media [^{14}C]arachidonate, and PGE accumulation in 1,650 mosM buffer containing a high

TABLE VII

Effects of Urea on Ca^{2+}-Stimulated Slice cAMP and Media [^{14}C]Arachidonate and PGE Accumulation in Inner Medullary Incubates

Total osmolality	Media composition							
			cAMP		^{14}C Accumulation rate		PGE	
	Na^+	Urea	$-Ca^{2+}$	$+Ca^{2+}$	$-Ca^{2+}$	$+Ca^{2+}$	$-Ca^{2+}$	$+Ca^{2+}$
mosM	*mM*	*mM*	*pmol/mg tissue*		*dpm/min/g tissue*		*ng/mg tissue*	
1,650	365	900	0.34±0.04	0.39±0.04	434±58	677±98	3.41±0.56	4.24±0.63
1,650	817	0	1.56±0.06‡	2.19±0.31*‡	756±62‡	1,926±286*‡	6.72±0.93‡	13.1±1.9*‡
1,205	145	900	0.26±0.04	0.37±0.04	467±64	691±88	2.98±0.44	3.61±0.52

All inner medullary slices were preincubated for 20 min at 37°C in Ca^{2+}- and Mg^{2+}-free 1,650 mosM buffer containing 5 μM A23187 and 2 mM EGTA. Slices in ^{14}C studies were prelabeled as described in Table VI. Slices were then washed and transferred to media containing 2 mM EGTA and 5 μM A23187. Total osmolality, Na^+, and urea concentrations of these media are shown. The incubation was then continued for an additional 25 min. Slices were then transferred to fresh buffer of the same composition as that during the second period and the incubation continued for an additional 35 min. Where shown, 3.5 mM Ca^{2+} was present for the final 20 min of the third incubation. Values represent mean±SE of determinations from four separate experiments.

* $P < 0.01$ compared to corresponding value in the absence of Ca^{2+}.

‡ $P < 0.01$ comparing 1,650 mosM buffer without urea to 1,650 or 1,205 mosM buffer with 900 mM urea.

concentration of NaCl without urea. This differs from the action of Ca^{2+} in either 1,650 or 1,205 mosM buffer containing 900 mM urea. Before Ca^{2+} addition, basal levels of all parameters were also higher in 1,650 mosM buffer containing excess NaCl but devoid of urea, than in either 1,650 or 1,205 mosM buffer with urea.

Table VIII compares the effects of urea on cAMP and PGE accumulation in the presence and absence of Ca^{2+} to those of mannitol, sucrose, and NaCl. In contrast to the actions of urea, hypertonic buffers containing mannitol, sucrose, or NaCl alone as the dominant solute stimulated both tissue cAMP and media PGE accumulation compared to slices incubated at 305 mosM. Moreover, the stimulatory effects of these solutes were expressed in the absence of extracellular Ca^{2+}. The accumulation of PGE observed in the presence of extracellular Ca^{2+} plus A23187 in combination with either hypertonic mannitol, sucrose, or NaCl was significantly (NaCl) or slightly greater than observed with each stimulus alone. However, the effects of combination of these agents on PGE accumulation were not clearly additive in any instance (Table VIII). The concentration of each agent employed alone gave maximal stimulation.

TABLE VIII

Effects of Impermeant Solutes vs. Urea on Slice cAMP and Media PGE Accumulation in Inner Medullary Incubates

Solute added	Total osmolality	$-Ca^{2+}$	$+Ca^{2+}$
cAMP, *pmol/mg tissue*	*mosM*		
None	305	0.43±0.05	2.78±0.35‡
Urea	1,650	0.32±0.04	0.40±0.05*
Mannitol	1,650	2.86±0.35*	3.17±0.41
Sucrose	1,650	2.36±0.29*	2.95±0.37
NaCl	1,650	1.89±0.21*	2.63±0.32
PGE, *ng/mg tissue*			
None	305	4.62±0.59	13.9±1.8‡
Urea	1,650	3.94±0.51	4.59±0.63*
Mannitol	1,650	14.2±1.8*	17.3±2.2
Sucrose	1,650	13.4±1.8*	16.5±1.9
NaCl	1,650	9.37±1.1*	15.9±1.8‡

Slices were initially incubated for 20 min as described in Table VII, and then transferred to buffers with the total osmolalities shown for a final 35-min incubation. Hypertonic buffers were prepared by addition of urea, mannitol, sucrose, or NaCl alone to standard 305 mosM Krebs buffer. Where shown, Ca^{2+} was present for the final 20 min of the second incubation. Values represent mean±SE of determinations from three separate experiments.

* $P < 0.01$ compared to corresponding value at 305 mosM.

‡ $P < 0.01$ compared to corresponding value in the absence of Ca^{2+}.

DISCUSSION

A reciprocal relationship exists between inner medullary cAMP accumulation and media osmolality, which is expressed over a wide range of O_2 availability (1) and is rapidly reversible (Table I, Fig. 1). This relationship pertains in either the presence or absence of IBMX, a potent inhibitor of cyclic nucleotide phosphodiesterase activity. Therefore, the changes most likely reflect osmotic effects on cAMP generation. The current data indicate that the stimulatory actions of reduced media osmolality on inner medullary cAMP accumulation are in a large part Ca^{2+} dependent. Moreover, Ca^{2+} deprivation lowers inner medullary cAMP in tissue incubated at 750 mosM, an effect which is specific relative to Mg^{2+} or K^+ deprivation, and clearly reversible. The latter findings and the ability of several agents, including AVP, to stimulate cAMP accumulation in Ca^{2+}-free buffer indicate that the reduction in cAMP observed in Ca^{2+}-free 750 mosM buffer was not a result of tissue injury or ATP depletion under these incubation conditions.

The renal inner medulla is an active site of prostaglandin synthesis (5–9, 23, 25–27) and there is evidence that Ca^{2+} may play a role in the regulation of this process in kidney (28, 29), and in platelets (28). Several observations in the current study suggest that the Ca^{2+}-dependent effects of osmolality on inner medullary cAMP are linked to alterations in Ca^{2+}-responsive prostaglandin synthesis. Thus, addition of Ca^{2+} to Ca^{2+}-deprived inner medulla with or without prior exposure to A23187 increased both tissue cAMP and media PGE accumulation at 750 mosM, whereas Ca^{2+} had no significant effect on either parameter at 1,650 mosM (Table III). The ability of Ca^{2+} or Ca^{2+} plus A23187 to stimulate tissue cAMP or media PGE accumulation at 750 mosM is consistent with earlier observations of Ca^{2+}-dependent changes in these parameters when inner medulla is incubated in a standard low osmolality buffer system (28–30). Addition of indomethacin and meclofenamate, inhibitors of cyclooxygenase activity (31), or exclusion of O_2, which is required as a cosubstrate with arachidonate for prostaglandin generation (32), blocked both Ca^{2+}-responsive cAMP and PGE accumulation in 750 mosM buffer. Moreover, arachidonate markedly increased cAMP in complete and Ca^{2+}-free 750 mosM or 1,650 mosM buffer, but was ineffective in the presence of indomethacin or in the absence of O_2. By contrast, exogenous PGE_2 increased cAMP in the presence or absence of Ca^{2+}, O_2, or indomethacin in both 750 and 1,650 mosM buffer. Although other interpretations are not excluded, one mechanism supported by these results was an action of high osmolality to suppress Ca^{2+}-responsive cAMP accumulation by

blocking Ca^{2+}-responsive prostaglandin generation. The availability of free arachidonate in tissue may be rate limiting to prostaglandin synthesis (29, 33, 34). Ca^{2+}-responsive release of prostaglandins has been demonstrated in platelets and inner medulla, and postulated to be linked to Ca^{2+}-responsive acyl hydrolase activity (28, 29). The clear correlation between the effects of Ca^{2+} plus A23187 on [^{14}C]arachidonate release and PGE synthesis in inner medulla (Tables V and VI) are compatible with this mechanism of Ca^{2+} action. The ability of exogenous arachidonate to increase cAMP and PGE accumulation in Ca^{2+}-deprived inner medulla at 750 and 1,650 mosM also suggested a role for limited arachidonate availability in the reductions in PGE and cAMP accumulation observed in the absence of Ca^{2+}, and at high osmolality. This possibility was supported by the observation that Ca^{2+} augmentation of [^{14}C]arachidonate release from prelabeled inner medullary slices was markedly decreased at 1,650 compared to 750 mosM. An effect of Ca^{2+} on fatty acid release was not observed at either osmolality when slices were prelabeled with [^{14}C]palmitate rather than arachidonate. Moreover, tetracaine and mepacrine reduced or blocked Ca^{2+} augmentation of arachidonate release and Ca^{2+} stimulation of PGE and cAMP observed at 750 mosM. Tetracaine has been shown to block Ca^{2+} binding and uptake in other tissues (35, 36), and thus may have inhibited Ca^{2+} actions in inner medulla by diverse effects on Ca^{2+} homeostasis. However, an action common to both tetracaine (37) and mepacrine (38) is inhibition of phospholipase A_2 activity, which demonstrates Ca^{2+} dependence in vitro (39, 40). Accordingly, Ca^{2+} augmentation of arachidonate release may reflect an action on phospholipase A_2 activity that is suppressed by either high osmolality or by agents such as tetracaine and mepacrine. An action of Ca^{2+} on phospholipase A_2 would be consistent with its selective effect on arachidonate vs. palmitate release (41), and with current evidence implicating tissue phospholipids as a major source of free arachidonate utilized in prostaglandin synthesis in inner medulla (22, 23, 25) and platelets (15). However, inner medullary triglycerides also contain considerable arachidonate (25) and this or other endogenous sources of the fatty acid are not excluded by the present study.

The inhibition of Ca^{2+}-responsive arachidonate release, PGE synthesis, and cAMP accumulation observed in hypertonic buffer in the present study reflected predominantly an action of urea (Table VII). Marked stimulation of prostaglandin synthesis by high osmolality has previously been demonstrated in studies of rat renal medulla slices (7), cultured medullary interstitial cells (9), and intestine (42). However, it is notable that the most striking effects were observed with solutes other than urea (NaCl, sucrose, mannitol). By contrast high osmolality buffer containing urea has been found to produce only slight stimulation (7, 42), no effect (8), or inhibition (6) of prostaglandin synthesis. The injection of NaCl into the renal artery of dog also resulted in increased PGE release, whereas equiosmolar urea was ineffective (26). The present observations indicate that the solute employed is a critical determinant of the effects of hyperosmolality on prostaglandin synthesis. Thus, in agreement with earlier observations (7, 9), hypertonic mannitol, sucrose, or NaCl alone stimulated PGE accumulation compared to values observed in either equiosmolar buffer containing urea or in 305 mosM buffer (Table VIII). The enhancement of PGE accumulation in response to mannitol, sucrose, or NaCl was accompanied by an increase in cAMP accumulation. Both of these stimulatory actions were well expressed in the absence of extracellular Ca^{2+}. The effect of hypertonic NaCl to increase PGE accumulation in inner medullary incubates in the absence of extracellular Ca^{2+} are consistent with prior observations (43). Despite the apparently Ca^{2+}-independent actions of hypertonic mannitol, sucrose, and NaCl, the stimulation of PGE or cAMP accumulation by these solutes was not clearly additive with that of extracellular Ca^{2+} plus A23187. Therefore, the stimuli effective in the absence of extracellular Ca^{2+} do not necessarily increase PGE accumulation through separate pathways or through effects on different cell populations in the inner medulla. It has previously been proposed that increased cytosolic Ca^{2+} triggers stimulation of PGE synthesis by inner medulla in response to either Ca^{2+} plus A23187 or hypertonicity, with solutes acting by the mobilization of Ca^{2+} from intracellular stores (43). The nonadditive effects of Ca^{2+} plus A23187 and hypertonic mannitol, sucrose, or NaCl are consistent with a common final pathway such as Ca^{2+}, but other possibilities are not excluded. However, as demonstrated by the current results, urea, the dominant solute of inner medulla in vivo, significantly suppresses at least the Ca^{2+}-responsive component of prostaglandin synthesis, an action which is strikingly different from the stimulatory effects of the three relatively impermeant solutes tested. Although the mechanism by which urea exerts its inhibitory action on prostaglandin synthesis remains uncertain, an effect to limit arachidonate availability is strongly implicated by the current results (Table V, Fig. 2). General considerations would include interference with Ca^{2+} binding or transport by urea, reduction in acyl hydrolase activity, or reduction in cellular lipid pools containing arachidonate. Inhibitory effects because of reduced cell volume at high urea concentrations seem unlikely in view of the fact that mannitol, sucrose, and NaCl, which are more impermeant solutes than urea and thus expected to

produce greater reductions in cell volume at an equivalent osmolality, stimulated PGE accumulation. If urea directly interferes with Ca^{2+} binding or movement, this action is not readily reversed by increasing extracellular Ca^{2+}, because 6 mM Ca^{2+} failed to stimulate cAMP, PGE, or arachidonate release at 1,650 mosM (900 mM urea).

Whatever the precise mechanism, enhancement of inner medullary prostaglandin synthesis at low osmolality (urea) may help to explain some in vivo alterations in renal excretion of prostaglandins. Thus, recent reports have described increased urinary excretion of prostaglandins during osmotic and/or water diuresis in the dog (44, 45) and man (46), as well as after administration of the loop diuretics, furosemide, and ethacrynic acid (45). These effects could be a direct consequence of stimulation of inner medullary prostaglandin synthesis by the resultant fall in medullary tonicity known to occur in these settings. Similarly, the enhanced urinary excretion of PGE reported in Bartter's syndrome and in other polyuric states (47, 48) may also be linked to reductions in medullary tonicity. Bartter and co-workers (49) and others (50) have recently presented evidence implicating defective chloride reabsorption in the thick ascending loop of Henle as a proximal lesion in patients with Bartter's syndrome. The reduction in medullary osmolality resulting from such a lesion may contribute to the enhanced renal prostaglandin synthesis and excretion seen in this disorder. Previous studies have demonstrated that vasopressin stimulation of inner medullary soluble protein kinase activity is suppressed at low osmolality (1, 2). Inhibitors of prostaglandin synthesis increase the sensitivity of protein kinase to vasopressin in vitro (1) and have been reported to potentiate vasopressin-mediated increases in cAMP and the hydroosmotic actions of vasopressin in vivo (51, 52). Thus, it is possible that stimulation of prostaglandin synthesis at low osmolality contributes to both increased urinary prostaglandin excretion (44–48) and reduced vasopressin sensitivity (10, 53, 54) associated with some polyuric states. Because previous studies from our own and other laboratories have found the effects of PGE_2 and vasopressin on inner medullary adenylate cyclase, cAMP and/or protein kinase to be additive (1, 55), the product of the prostaglandin synthetic pathway responsible for reduced vasopressin sensitivity at low osmolality is not necessarily PGE_2.

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