

# Renal Inner Medullary Prostaglandin Synthesis

## A CALCIUM-CALMODULIN-DEPENDENT PROCESS

### SUPPRESSED BY UREA

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**ABSTRACT** Previous studies have demonstrated that hyperosmolar NaCl and mannitol stimulate immunoreactive prostaglandin E (iPGE) production by slices of inner medulla (IM), whereas urea inhibits this process. In the present study, the roles of  $\text{Ca}^{2+}$  and calmodulin in the control of PGE synthesis in IM and the basis for the differential actions of solutes were examined. A23187 increased [ $^{14}\text{C}$ ]arachidonate (AA) release and iPGE accumulation in the presence but not in the absence of media  $\text{Ca}^{2+}$  whereas stimulation by hypertonic NaCl or mannitol was well expressed with  $\text{Ca}^{2+}$  or in  $\text{Ca}^{2+}$ -free buffer containing 2 mM EGTA. Hypertonic urea and trifluoperazine (TFP), an inhibitor of actions of the  $\text{Ca}^{2+}$ -CaM complex, suppressed increases in [ $^{14}\text{C}$ ]AA release and iPGE induced by A23187, NaCl, or mannitol. By contrast, increases in iPGE in response to exogenous AA were not altered by urea or TFP.  $\text{Ca}^{2+}$  (25–100  $\mu\text{M}$ ) increased acyl hydrolase (AH) activity in EGTA washed (4°C) 100,000 g particulate fractions of IM threefold, thereby restoring AH activity to the higher basal values of particulate fractions not washed with EGTA. This action of  $\text{Ca}^{2+}$  was blocked by hypertonic urea or TFP, whereas AH activity was not influenced by NaCl or mannitol in the presence or absence of  $\text{Ca}^{2+}$ . In contrast to their effects on AH activity, hypertonic urea and TFP did not alter conversion of AA to  $\text{PGE}_2$ ,  $\text{PGF}_{2\alpha}$ , or  $\text{PGD}_2$  by IM microsomal fractions.  $\text{Ca}^{2+}$ -induced increases in particulate AH were blunted after partial depletion of endogenous CaM-like activity.  $\text{Ca}^{2+}$  action was restored by addition of purified exogenous CaM, but not by addition of other small acidic proteins, including troponin C. The findings support a role for CaM in the regulation of PGE synthesis in the IM at the level of  $\text{Ca}^{2+}$ -responsive AH activity. They further imply that urea suppresses PGE

synthesis in IM through inhibition of AH and a reduction in the availability of endogenous AA for conversion to PGE.

## INTRODUCTION

Observations from several laboratories have indicated that prostaglandin (PG)<sup>1</sup> synthesis in the renal inner medulla (IM) and in other tissues is a  $\text{Ca}^{2+}$ -responsive process (1–3). The influence of  $\text{Ca}^{2+}$  on PG synthesis may be mediated through an action of this cation to stimulate the release of free arachidonate (AA) from tissue lipid stores (1), a step which appears to be rate limiting in the cellular generation of PG (4). In the renal IM, where osmolality ordinarily varies over a wide range under normal physiologic conditions (5), there is evidence that solute concentration is an important local determinant of PG synthesis (1, 6–8). Earlier studies from our laboratory indicated that effects of solute concentration on immunoreactive PGE (iPGE) synthesis, analogous to those of  $\text{Ca}^{2+}$ , are mediated through alterations in the release of AA from tissue lipid stores (1). However, the influence of osmolality on inner medullary iPGE generation is complex and responses differ qualitatively with the specific solute tested (1). Thus, whereas hypertonic NaCl or mannitol stimulate inner medullary iPGE synthesis, urea suppresses the increases in iPGE synthesis induced by  $\text{Ca}^{2+}$ , hypertonic NaCl, or mannitol (1). When IM of rat is exposed concurrently to a combination of hypertonic urea and NaCl in concentrations that pertain in this region of the kidney during hydropenia, the inhibitory effects of urea are expressed (1).

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<sup>1</sup>Abbreviations used in this paper: AA, arachidonic acid, arachidonate; AH, acyl hydrolase; CaM, calmodulin; IM, renal inner medulla; iPGE, immunoreactive prostaglandin E. KRBC, Krebs Ringer bicarbonate buffer containing 5 mM glucose; PG, prostaglandin; PMZ, promethazine; TFP, trifluoperazine.

In the present study, we examined the mechanisms by which  $\text{Ca}^{2+}$ , hypertonic NaCl, and mannitol stimulate iPGE synthesis in rat IM and the basis for the differential action of urea on this process, relative to those of NaCl or mannitol. Since calmodulin (CaM) has been implicated in the expression of  $\text{Ca}^{2+}$  actions on a number of cellular processes and enzymes (9), including platelet phospholipase activity (10), the role of CaM in the regulation of  $\text{Ca}^{2+}$ -responsive inner medullary iPGE synthesis was also examined.

## METHODS

**Preparation of tissue.** Sprague-Dawley rats weighing 350–400 g (female retired breeders) were obtained from Zivic-Miller Laboratories, Inc., Allison Park, Pa. Rats were fasted for 18 h before study but allowed water ad lib. Rats were anesthetized with pentobarbital (5 mg/100 g body wt, i.p.), the kidneys excised and chilled in 0.85% NaCl at 0–4°C. The renal capsules were removed and slices of IM prepared with a Stadie Riggs microtome (Arthur H. Thomas Co., Philadelphia, Pa.) as previously described (1).

Inner medullary slices (20–30 mg/flask) were incubated in 25-ml flasks with 2 ml of Krebs-Ringer bicarbonate buffer (total osmolality, 305 mosmol/liter; total  $\text{Na}^+$ , 145 mM;  $\text{HCO}_3^-$ , 25 mM;  $\text{Ca}^{2+}$ , 1.5 mM;  $\text{Mg}^{2+}$  1.2 mM, pH 7.4) containing 5 mM glucose (KRBG) or in KRBG whose osmolality was increased by the addition of urea, NaCl, or mannitol to give the final concentration indicated in the text. All buffers were equilibrated with 5%  $\text{O}_2$ , 5%  $\text{CO}_2$  balance  $\text{N}_2$  before use. This mixture was used as the standard gas phase for the inner medullary incubates for reasons previously described (1).

**Extraction and assay of iPGE content.** iPGE content of the slice incubation media was assessed by radioimmunoassay according to the procedures of Stylos et al. (11), using antiserum commercially obtained (Regis Chemical Co., Morton Grove, Ill). [ $^3\text{H}$ ]PGE<sub>2</sub> (1,500 cpm) was added to 1 ml of media before storage at –20°C. Acidified samples (pH 3–3.5) were extracted in  $\text{CHCl}_3$ , purified by silicic acid chromatography, and assayed for iPGE content within 2 wk, as previously described (1). In preliminary experiments EGTA (2 mM), indomethacin (50  $\mu\text{M}$ ), promethazine (0.5 mM), or trifluoperazine (0.5 mM) did not interfere with the PGE assay. the PGE assay.

**Determination of accumulation of [ $^{14}\text{C}$ ]arachidonate in the media of inner medullary slices prelabeled with [ $^{14}\text{C}$ ]arachidonate (AA).** Approximately 100 mg of inner medullary slices were incubated at 37°C for 30 min in 2 ml of complete KRBG, 1.5 mM  $\text{Ca}^{2+}$ , and 0.5  $\mu\text{Ci}$  of [ $^{14}\text{C}$ ]AA. At the end of 30 min, the slices were washed briefly and transferred to fresh KRBG plus 1 mg/ml fatty acid-free bovine serum albumin. Slices were transferred at 30-min intervals to KRBG or KRBG whose osmolality had been increased by the addition of urea, NaCl, or mannitol as indicated in the text. As shown,  $\text{Ca}^{2+}$  was present (2 mM EGTA, 3.5 mM  $\text{Ca}^{2+}$ ) or excluded (2 mM EGTA) from the final two 30-min incubations. A23187 (5  $\mu\text{M}$ ) was added to some incubates during the final 15 min of incubation. [ $^{14}\text{C}$ ]AA accumulation was calculated from the media counting rate during the 10-min period immediately after transfer of slices to a high osmolality buffer or addition of A23187.

In some experiments, media or tissue lipids were separated into major classes by silicic acid chromatography, and the distribution of  $^{14}\text{C}$  among neutral lipids, phospholipids, and prostaglandins determined as previously described (1). The neutral lipid fraction was further fractionated by thin-layer

chromatography in hexane/ethyl ether/acetic acid (80:20:2) on plates of silica gel G (Redi-Coat G) (1), or on silver nitrate-impregnated thin-layer plates (12). Recovery of sample radioactivity was monitored at each step during lipid extraction and analysis and varied between 80–82% (tissue lipids) and 87–92% (media lipids). Extraction of tissue lipids from slices incubated in KRBG before exposure to high osmolality buffer, indicated that  $32 \pm 6\%$  of the label was in the neutral lipid fraction,  $67 \pm 5\%$  was present as phospholipid, and 0–0.4% as prostaglandin. Chromatographic analysis of lipid extracts of media, sampled at the end of the incubation, indicated that accumulated  $^{14}\text{C}$  was present predominantly as free fatty acid ( $82 \pm 9\%$ ), with lesser amounts of prostaglandin ( $7 \pm 3\%$ ), and phospholipid ( $11 \pm 2\%$ ). Fatty acids were further separated by thin-layer chromatography according to degree of unsaturation following methylation with 14%  $\text{BCl}_3$  in methanol under  $\text{N}_2$  for 1 h at 80°C. The methylated lipid extracts and methylated fatty acid standards were applied to plates of Redi-Coat AG and the plates developed with 20% diethyl ether in petroleum ether (12). The spots were visualized with iodine vapor, scraped, and counted. Greater than 95% of the labeled free fatty acid released cochromatographed with authentic methyl arachidonate.

**Assay of acyl hydrolase (AH) activity.** Renal inner medullary slices (100 mg/2 ml media per flask, 2.5 g total tissue wet wt) were incubated with [ $^{14}\text{C}$ ]AA (0.5  $\mu\text{Ci}$ ) in complete KRBG for 2 h at 37°C, then transferred to KRBG plus 5 mg/ml fatty acid free albumin and incubated for another 20 min. Slices were homogenized in 50 mM Tris, pH 7.4, 5 mg/ml fatty acid free albumin, or the same buffer plus 10 mM EGTA, and centrifuged at 10,000 g for 20 min. The 10,000 g supernate was then centrifuged at 100,000 g for 60 min and washed once by centrifugation. The 100,000 g particulate fraction was resuspended for assay in 50 mM glycine, pH 9.0, in the presence or absence of 10 mM EGTA. Assays for the determination of AH activity routinely contained at final concentration 50 mM glycine (pH 9.0), with or without 10 mM EGTA, and test agents, as indicated in the text. When  $\text{CaCl}_2$  was added with EGTA, the final concentration of  $\text{Ca}^{2+}$  was that present in excess of 10 mM EGTA. Assays were conducted in a final volume of 1.5 ml for 20 min at 37°C. In addition, a zero time sample was prepared but not incubated. Reactions were stopped by acidification to pH 3–3.5, with HCl and extracted with 3 vol of 2:1  $\text{CHCl}_3/\text{CH}_3\text{OH}$ . The distribution of  $^{14}\text{C}$  label among neutral lipid, phospholipid, and prostaglandin fractions was determined by silicic acid column chromatography as previously described (1). Phospholipids were further fractionated by thin-layer chromatography as previously reported (1). [ $^{14}\text{C}$ ]Fatty acid was routinely isolated and counted following thin-layer chromatography in hexane/ethyl ether/acetic acid (80:20:2). In some experiments, fatty acids were further fractionated by chromatography on silver nitrate-impregnated thin-layer plates (12). Recoveries were monitored at each stage of lipid extraction and analysis and found to vary between 85 and 95%. Extraction and analysis of the zero time samples, as described above, indicated that ~20–25, 1–2, and 75–80% of the total recovered  $^{14}\text{C}$  counts were present in the neutral lipid, prostaglandin and phospholipid fraction, respectively. Approximately 40–44, 18–22, and 35–40% of the counts recovered in the phospholipid fraction were present as phosphatidyl choline, phosphatidyl ethanolamine, and phosphatidyl inositol, respectively. Treatment of the phospholipid fraction with phospholipase A<sub>2</sub> resulted in the release of 90–95% of the counts present in phospholipid as free fatty acid, indicating that the majority of the [ $^{14}\text{C}$ ]AA that was incorporated into phospholipid was present in the two position. Before incubation, ~1–4% of the counts present in the neutral lipid fraction (0.25–1% of total

counts) cochromatographed with authentic free fatty acid. These counts were routinely subtracted from the sample counts. After incubation of particulate fractions for the determination of AH activity, no more than 30% of the counts in the neutral lipid fraction (6% of total counts) were released. Under all conditions of incubation described >95% of the released free fatty acid was AA. Results are expressed as disintegrations per minute of  $^{14}\text{C}$  released as free fatty acid per minute per milligram protein. Reaction rates were linear with time for at least 25 min and with protein concentration of 0.5–2 mg/assay. Consistent with studies of particulate AH of platelets (13), fatty acid release rates were much lower when an exogenous labeled phospholipid,  $\text{L}\alpha$ -palmitoyl-[2-oleoyl-1- $^{14}\text{C}$ ]phosphoryl choline, was used as substrate for hydrolysis by inner medullary microsomes. The low basal rates of fatty acid release precluded examination of potential inhibitor effects. Thus, microsomes with labeled endogenous lipid pools were used in the present studies.

**Assay of phospholipase  $A_2$ .** Phospholipase  $A_2$  activity was determined from the release of free [ $^{14}\text{C}$ ]oleate from  $\text{L}\alpha$ -palmitoyl [2-oleoyl-1- $^{14}\text{C}$ ]phosphoryl choline. The phospholipid substrate was prepared as an emulsion with egg yolk lecithin by dissolving 0.8 mg of egg yolk lecithin and 0.5  $\mu\text{Ci}$  of  $\text{L}\alpha$ -palmitoyl [2-oleoyl-1- $^{14}\text{C}$ ]phosphoryl choline (55 mCi/mmol) in 50  $\mu\text{l}$  of ether, followed by the addition of 1 ml of water. The mixture was sonicated 2 $\times$  for 30 min each at the highest setting in an Artek probe sonic dismembrator. Reaction mixture contained at final concentration 50 mM glycine, pH 9.0, 0.2 mM egg yolk lecithin,  $\text{L}\alpha$ -palmitoyl-[2-oleoyl-1- $^{14}\text{C}$ ]phosphoryl choline. Soluble phospholipase  $A_2$  activity, extracted from *Crotalus adamanteus* snake venom, was added to the assay mixture. Reactions were conducted for 10 min at 37°C. In addition, a zero time sample was prepared but not incubated. Reactions were stopped by adjusting the pH to 3–3.5 with HCl followed by extraction with 3 vol of 2:1  $\text{CHCl}_3/\text{CH}_3\text{OH}$ . The distribution of  $^{14}\text{C}$ -label among neutral lipid, phospholipid, and prostaglandin fractions was determined by silicic acid chromatography, as described above. In some experiments, the neutral lipid fraction was further fractionated as described above. Virtually all the counts recovered in the neutral lipid fraction cochromatographed on thin-layer plates with authentic oleic acid. Accordingly, the counts recovered in the neutral lipid fraction following silicic acid column chromatography were used as a measure of phospholipase  $A_2$  activity. The disintegrations per minute in the neutral lipid fraction of the zero time sample (<1% of the total added disintegrations per minute) were subtracted from those in the samples. Under assay conditions the release of [ $^{14}\text{C}$ ]fatty acid (4–20% of the total dpm) was linear with time for 5–20 min and with an enzyme activity of 0.001–0.005 U/assay. 1 U is defined as that amount of enzyme which hydrolyzes 1  $\mu\text{mol}/\text{min}$  of phosphatidyl choline.

**Determination of prostaglandin synthetase activity.** Rat inner medullary slices (500 mg/2.5 ml) were homogenized in 0.1 M potassium phosphate, pH 8.0, and centrifuged at 10,000 g for 20 min. The 10,000 g supernate was centrifuged at 100,000 g. The 100,000 g pellet was resuspended in 2.5 ml of 0.1 M phosphate, pH 8.0. Reaction mixtures for determination of PG synthetase activity were the same as those described by Schwartzman et al. (14), and contained 5  $\mu\text{M}$  arachidonic acid, 40,000 dpm of [ $^{14}\text{C}$ ]AA, and 0.8–1.25 mg of microsomal protein in a final volume of 2 ml. Mixtures were incubated with shaking for 30 min at 37°C in 25-ml flasks open to the atmosphere. Reactions were stopped by acidification to 3–3.5 with HCl and extracted with 3 vol of 2:1  $\text{CHCl}_3/\text{CH}_3\text{OH}$ . The distribution of  $^{14}\text{C}$  among AA,  $\text{PGE}_2$ ,  $\text{PGF}_{2\alpha}$ , and  $\text{PGD}_2$  was determined by thin-layer chromatography

on plates of silica gel G with an organic layer of ethyl acetate/acetic acid/isooctane/ $\text{H}_2\text{O}$ , 11:2:5:10. Authentic standards were applied over each sample spot. Lipids were visualized with  $\text{I}_2$  vapor, the spots transferred to vials, and counted by liquid scintillation spectrometry.

**Determination of CaM.** CaM-like activity was determined in the 100,000 g particulate fraction of renal medullary homogenates by its ability to stimulate activator-deficient 3',5' cyclic AMP-phosphodiesterase activity in the presence of  $\text{Ca}^{2+}$  (15). The particulate fraction was heated at 95° for 5 min to destroy endogenous phosphodiesterase activity before addition to the assay mixture. A modification of the two-step procedure for assay of phosphodiesterase activity developed by Thompson and Appleman (16) was used as previously described (17). Reaction mixtures contained 40 mM HCl, Tris pH 7.6, 1.3 mM dithiothreitol, 0.3 mg/ml bovine serum albumin, 5 mM  $\text{MgSO}_4$ , 1 mM EGTA, 0.1 mM cAMP, 0.0008 U/ml of activator-deficient phosphodiesterase activity, and when present, 20–80  $\mu\text{g}$  of protein from the heat-treated 100,000 g particulate fraction in a final volume of 0.5 ml. Where indicated,  $\text{Ca}^{2+}$  (100  $\mu\text{M}$  in excess of EGTA) was added to the enzyme assay mixture. The reaction was initiated by the addition of activator-deficient phosphodiesterase activity and was conducted for 5 min at 30°C. The reaction was terminated by boiling for 1 min followed by addition of 50  $\mu\text{g}/\text{ml}$  of snake venom (*Ophiophagus hannah*) and 2,000 cpm of [ $^{14}\text{C}$ ]adenosine to assess recovery. After incubation with snake venom for 30 min at 30°C, AG1-X2 was added to remove unreacted substrate. The samples were centrifuged at 2,500 g for 20 min and an aliquot of the supernatant fluid was counted in a liquid scintillation counter. Recoveries of [ $^{14}\text{C}$ ]adenosine were ~60%, and the results were corrected accordingly. Endogenous phosphodiesterase activity in the heat-treated 100,000 g particulate fraction was negligible. In the absence of  $\text{Ca}^{2+}$ , CaM-like activity was not detectable in the 100,000 g particulate fraction. A standard curve was constructed using a homogeneous preparation of CaM. CaM-like activity of the particulate fraction was expressed as micrograms per milligram of protein. Activity was linear for at least 10 min with added CaM-like activity 20–80  $\mu\text{g}$ , and with added purified CaM 15–60 ng.

Protein was determined by the method of Lowry et al. (18). Statistical significance of differences between mean values was determined by Student's *t* test for unpaired values (19). Unless otherwise indicated, in studies of iPGE, each condition in a given experiment was represented by triplicate incubation flasks, whereas in studies of [ $^{14}\text{C}$ ]AA release, AH activity, or CaM-like activity, each incubation was performed in duplicate. For statistical analysis, the average value derived from replicate sets of flasks from the same experiment was entered as a single value; degrees of freedom is 4 or 6 comparing value for any two experimental conditions derived from three or four experiments by *t* test for unpaired values.

**Materials.**  $\text{PGE}_2$ ,  $\text{PGF}_{2\alpha}$ , and  $\text{PGD}_2$  were gifts of the Upjohn Co., Agricultural Prods MKT. Kalamazoo, Mich. Tri-fluoperazine (TFP) and promethazine (PMZ) were gifts of Smith Kline and French Laboratories, Philadelphia, Pa., and Wyeth Laboratories, Philadelphia, Pa., respectively. [ $^{14}\text{C}$ ]AA (50 mCi/mmol) was obtained from Amersham-Corp., Arlington Heights, Ill. Phospholipase  $A_2$  (*Crotalus Adamanteus*), activator-deficient phosphodiesterase activity, and horse heart cytochrome C, type VI, were obtained from Sigma Chemical Co., St. Louis, Mo. Ribonuclease A was obtained from Pharmacia Diagnostics, Div. of Pharmacia, Inc. Piscataway, N. J. Phospholipid standards Redi-Coat AG and Redi-Coat G thin-layer plates were purchased from Supelco, Inc., Supelco Park, Bellefonte, Pa. AA was obtained from Nu-

Chek Prep, Elysian, Minn.  $\alpha$ -palmitoyl [2-oleoyl-1- $^{14}$ C]-phosphoryl choline was obtained from Applied Science Labs, Inc., State College, Pa. Purified CaM, prepared from bovine adrenal medulla, and troponin C were the kind gifts of Dr. Carole Coffee, University of Pittsburgh, Pa. The sources of all other chemicals have previously been reported (1, 6).

## RESULTS

**Effects of solutes and  $\text{Ca}^{2+}$  on [ $^{14}$ C]AA release.** Table I shows the effects of hypertonic urea, NaCl, and mannitol (1,000 mosmol/l on [ $^{14}$ C]AA release into the media of inner medullary slice incubates. Hypertonic mannitol, or NaCl, but not hypertonic urea, increased [ $^{14}$ C]AA release. Stimulation by NaCl and mannitol occurred in either  $\text{Ca}^{2+}$ -deplete or replete incubates, whereas A23187 increased [ $^{14}$ C]AA release in the presence (Table I) but not in the absence of  $\text{Ca}^{2+}$  (not shown). With  $\text{Ca}^{2+}$ , stimulation of [ $^{14}$ C]AA release by A23187 plus NaCl or mannitol was not additive (Table I). Moreover, urea suppressed increases induced by A23187 plus  $\text{Ca}^{2+}$ . The lowest concentration of urea that significantly suppressed [ $^{14}$ C]AA release from the slices in response to A23187 plus  $\text{Ca}^{2+}$  was 700 mosmol/liter (no urea,  $4,924 \pm 518$  dpm/min per g tissue; 700 mosmol/liter urea  $3,157 \pm 422$ ;  $n = 6$ ,  $P < 0.01$ ). The actions of these solutes or A23187 on [ $^{14}$ C]AA release under the incubation conditions shown in Table I were accompanied by qualitatively similar changes in media iPGE, as previously reported (1). In separate experiments, 800 or 1,600 mosmol/liter NaCl or mannitol were found to stimulate [ $^{14}$ C]AA release and iPGE to values not different from those observed at 1,000 mosmol/liter of the corresponding solute. Combined addition of 800 mosmol/liter urea with 800 mosmol/liter NaCl, or mannitol, however, suppressed significantly ( $\approx 50\%$ ) [ $^{14}$ C]AA release and iPGE compared with 800 or 1,600 mosmol/l NaCl or mannitol alone (not shown). By contrast, the marked increases in iPGE induced by 100  $\mu\text{M}$  exogenous AA occurred in  $\text{Ca}^{2+}$ -deplete or replete inner medullary incubates, were not inhibited by hypertonic urea, and were not additive with increases in iPGE induced by hypertonic NaCl, mannitol, or A23187 (1). These findings suggested that changes in iPGE induced by the solutes or A23187 were due to effects on AA availability.

**Effects of TFP on [ $^{14}$ C]AA and iPGE release.** Many of the biologic actions of  $\text{Ca}^{2+}$ , including activation of AH (10), are known to involve the intracellular  $\text{Ca}^{2+}$ -acceptor protein, CaM (9). To assess the possible role of CaM in regulation of inner medullary slice [ $^{14}$ C]AA release and iPGE synthesis, the effects on these processes of TFP, a potent phenothiazine inhibitor of the biologic action of the  $\text{Ca}^{2+}$ -CaM complex, were examined. Table II shows the actions of TFP, PMZ, a phenothiazine which does not inhibit  $\text{Ca}^{2+}$ -CaM action, and indomethacin, a cyclooxygenase inhibitor,

TABLE I  
Effects of Urea, NaCl, Mannitol, and A23187 on  
[ $^{14}$ C]AA Release by Inner Medullary Slices  
in the Presence and Absence of  $\text{Ca}^{2+}$

Final incubation conditions	$\text{Ca}^{2+}$		
	- $\text{Ca}^{2+}$		+ $\text{Ca}^{2+}$
	Additions		
	None	None	A23187
[ $^{14}$ C]AA release, dpm/min/g tissue			
KRBG, 305			
mosmol/l	1,097 $\pm$ 154	1,136 $\pm$ 125	5,352 $\pm$ 910†
+ Urea	1,073 $\pm$ 139	1,094 $\pm$ 164	1,324 $\pm$ 172*
+ NaCl	3,527 $\pm$ 564*	3,672 $\pm$ 681*	5,392 $\pm$ 593†
+ Mannitol	5,471 $\pm$ 985*	5,308 $\pm$ 902*	5,186 $\pm$ 726

For the determination of [ $^{14}$ C]AA release, inner medullary slices were incubated for 30 min with [ $^{14}$ C]AA as described in Methods. Slices were washed briefly, transferred to fresh KRBG containing 1 mg/ml fatty acid free albumin and the incubation continued for an additional 150 min. The media were changed at 30 min intervals. Where indicated,  $\text{Ca}^{2+}$  was present (3.5 mM  $\text{Ca}^{2+}$ , 2 mM EGTA) or excluded (2 mM EGTA) from the final two 30-min incubations. Some slices were exposed to KRBG whose osmolality was increased by the addition of urea, NaCl, or mannitol (1,000 mosmol/liter) for the final 30 min of incubation only. A23187, 5  $\mu\text{M}$  was added, where shown, for the final 15 min of incubation. The [ $^{14}$ C]AA accumulation shown was calculated from the media counting rate observed 10 min after transfer to a high osmolality buffer or addition of A23187. Results shown are means  $\pm$  SE of determinations pooled from four separate experiments.

\*  $P < 0.01$  compared to corresponding value in KRBG.

†  $P < 0.01$  compared to corresponding value with  $\text{Ca}^{2+}$  in the absence of A23187.

on basal [ $^{14}$ C]AA release and iPGE accumulation, and on the increases in these parameters induced by A23187 plus  $\text{Ca}^{2+}$ . Basal [ $^{14}$ C]AA release was not altered by TFP, PMZ, or indomethacin. Basal iPGE accumulation was suppressed to undetectable levels by indomethacin, but not changed by TFP or PMZ. TFP (25  $\mu\text{M}$ ) however, significantly suppressed increases in both [ $^{14}$ C]AA and iPGE accumulation induced by A23187. Inhibition of the responses of these parameters to A23187 was somewhat greater in the presence of 100 vs. 25  $\mu\text{M}$  TFP, whereas up to 100  $\mu\text{M}$  PMZ was without significant effect. Indomethacin (100  $\mu\text{M}$ ) suppressed A23187-induced iPGE accumulation to undetectable levels, but did not alter A23187 effects on [ $^{14}$ C]AA release.

Table III shows the actions of TFP, PMZ, and indomethacin on increases in [ $^{14}$ C]AA release and iPGE accumulation induced by hypertonic mannitol or NaCl. TFP (25  $\mu\text{M}$ ), but not 100  $\mu\text{M}$  PMZ or indomethacin, significantly inhibited increases in [ $^{14}$ C]AA release induced by hypertonic mannitol or

TABLE II  
Effects of TFP, PMZ and Indomethacin on Basal and A23187-stimulated [ $^{14}$ C]AA and  
iPGE Release into the Media of Inner Medullary Slice Incubates

Incubation conditions	[ $^{14}$ C]AA RELEASE		iPGE	
	None	A23187	None	A23187
	dpm/min/g tissue		ng/mg tissue	
Complete KRBC	1,042 $\pm$ 146	5,733 $\pm$ 688*	1.2 $\pm$ 0.2	4.2 $\pm$ 0.5*
+ 25 $\mu$ M TFP	1,287 $\pm$ 177	2,192 $\pm$ 289*†	1.0 $\pm$ 0.1	2.3 $\pm$ 0.3*†
+ 100 $\mu$ M TFP	1,322 $\pm$ 203	1,479 $\pm$ 161†	1.4 $\pm$ 0.2	1.6 $\pm$ 0.2†
+ 100 $\mu$ M Indomethacin	984 $\pm$ 123	5,398 $\pm$ 943*	ND	ND
+ 100 $\mu$ M PMZ	1,156 $\pm$ 198	4,623 $\pm$ 794*	1.1 $\pm$ 0.2	3.8 $\pm$ 0.6*

Inner medullary slices were incubated in complete KRBC, with media changes at 30-min intervals for a total of 180 min. [ $^{14}$ C]AA release and iPGE were determined as described in the footnote to Table I. Where shown, TFP, indomethacin, or PMZ were present for the final 2 h of incubation. As indicated, 5  $\mu$ M A23187 was present for the final 15 min of incubation only. Results shown are means  $\pm$  SE of nine determinations pooled from three separate experiments. ND, not detectable.

\*  $P < 0.01$  compared to corresponding value in the absence of A23187.

†  $P < 0.01$  compared to corresponding value in the absence of TFP, PMZ or indomethacin.

NaCl. TFP and indomethacin, but not PMZ, inhibited increases in iPGE accumulation induced by mannitol or NaCl. By contrast, TFP did not detectably alter increases in iPGE accumulation mediated by addition of exogenous AA, which were suppressed to undetectable levels by indomethacin.

**Effects of  $\text{Ca}^{2+}$ , solutes, and CaM on particulate AH activity.** Because observations from inner medullary slices implied that stimulation of iPGE, by  $\text{Ca}^{2+}$ , NaCl, and mannitol, and its inhibition, by urea and TFP, were due to effects on AA availability, the actions of these agents on AH activity were examined in subcellular preparations of IM. Studies in other tissues have demonstrated the presence of  $\text{Ca}^{2+}$ -dependent AH activity in plasma membranes and microsomes, the sites of enzyme activity most likely involved in PG synthesis (4). Accordingly, these subcellular fractions were examined.

Table IV shows AH activities of 100,000 g particulate fractions of IM prepared at 4°C with and without washing with 10 mM EGTA. Lipids of all preparations had been prelabeled with [ $^{14}$ C]AA. AH activity of particulate preparations not exposed to EGTA (I), determined as the rate of release of free [ $^{14}$ C]AA from lipid stores, was not altered by concentrations of  $\text{Ca}^{2+}$  or  $\text{Mg}^{2+}$  up to 5 mM. Basal AH activity of EGTA-treated preparations (II) was significantly reduced compared with that of untreated preparation I. Activity of the EGTA-treated preparation II was increased approximately threefold by addition of 100  $\mu$ M  $\text{Ca}^{2+}$  in excess of EGTA, and was restored to levels indistinguishable from those of preparation I. The lowest concentration of  $\text{Ca}^{2+}$  in excess of EGTA to increase particulate AH significantly was 25  $\mu$ M. The response to 5 mM  $\text{Ca}^{2+}$

was not different from that observed with 100  $\mu$ M. By contrast, up to 5 mM  $\text{Mg}^{2+}$  was without effect on the AH activity of the EGTA-treated fraction. Qualitatively similar  $\text{Ca}^{2+}$ -dependent AH activity was found in studies of partially purified plasma membranes prepared from rat IM (20).

The effects of hypertonic NaCl or urea on AH activity of the EGTA-washed particulate fractions II of IM are shown in Fig. 1. Increases in response to  $\text{Ca}^{2+}$  were comparable to those shown in Table IV. When tested over a concentration of 200–1,000 mosmol/liter, NaCl was without effect on AH activity in either the presence or absence of  $\text{Ca}^{2+}$ . Mannitol, over this same concentration range, was also without detectable effect on particulate AH (not shown). Urea, 200–1,000 mosmol/l, failed to alter AH activity in the absence of  $\text{Ca}^{2+}$  however, 600–1,000 mosmol/liter progressively suppressed  $\text{Ca}^{2+}$ -dependent AH activity (Fig. 1). Guanidine hydrochloride (1,000 mosmol/liter) produced qualitatively similar, but less suppression of  $\text{Ca}^{2+}$ -induced increases in particulate AH of preparation II (60% inhibition).

The lowest concentration of urea to produce statistically significant suppression of  $\text{Ca}^{2+}$ -induced increases in AH was 700 mosmol/l (no urea, 101  $\pm$  13 dpm/min per mg protein; 700 mosmol/liter urea, 65  $\pm$  8;  $n = 6$ ,  $P < 0.01$ ). Incubation of preparations I or II with 700–1,000 mosmol/liter urea for 30 min at 37°C in glycine assay buffer resulted in no additional loss of  $^{14}\text{C}$  from the neutral lipid or phospholipid fractions of the microsomes over that found in unincubated preparations or preparations incubated without urea. Distribution of label among the lipid fractions was not detectably altered during these incubations, whether

**TABLE III**  
*Effects of TFP, PMZ, and Indomethacin on Basal, Mannitol and NaCl-stimulated [<sup>14</sup>C]AA and iPGE Release into the Media of Inner Medullary Slice Incubates*

Incubation conditions	Additions		
	None	None	AA
	[ <sup>14</sup> C]AA release dpm/min/g tissue	iPGE ng/mg tissue	
KRBC	1,152±186	0.67±0.09	12.3±1.9
+ 25 μM TFP	1,387±164	0.74±0.13	10.2±1.4
+ 100 μM PMZ	1,324±141	0.71±0.11	11.6±2.2
+ 100 μM Indomethacin	1,056±177	ND	ND
KRBC + 1,000 mosmol/l mannitol	6,074±952*	9.2±1.3*	11.7±1.8
+ 25 μM TFP	2,136±307*†	2.7±0.62*†	10.5±1.4
+ 100 μM PMZ	5,367±778*	10.7±0.12*	9.0±1.3
+ 100 μM Indomethacin	5,879±921*	ND	ND
KRBC + 1,000 mosmol/l NaCl	4,872±752*	8.6±1.1*	11.3±1.6
+ 25 μM TFP	1,977±376*†	1.9±0.28*†	9.2±1.3
+ 100 μM PMZ	4,302±789*	7.2±0.9*	12.6±1.9
+ 100 μM Indomethacin	4,632±692*	ND	ND

Inner medullary slices were incubated in complete KRBC with media changes at 30-min intervals for a total of 180 min. [<sup>14</sup>C]AA and iPGE accumulation were determined as described in the footnote to Table I. Where indicated, some slices were exposed, for the final 30 min of incubation only, to media whose osmolality was increased by the addition of 1,000 mosmol/l NaCl or mannitol. Where stated, during the final 15 min of incubation, 100 μM AA was added. Where shown, TFP and indomethacin were present for the final 2 h of incubation. Results shown are means ±SE of values pooled from three separate experiments. ND, not detectable.

\*  $P < 0.01$  compared to value obtained in KRBC.

†  $P < 0.01$  compared to corresponding value obtained in the absence of TFP, PMZ, and indomethacin.

conducted in the presence or absence of urea. Thus, the inhibitory effects of urea on  $\text{Ca}^{2+}$ -induced increases in AH activity were not attributable to an action of urea to deplete labeled substrate from the particulate fraction. Moreover, in contrast to its action on  $\text{Ca}^{2+}$ -responsive particulate AH of IM, 1,000 mosmol/liter urea did not alter soluble phospholipase  $\text{A}_2$  activity (*Crotalus adamanteus*) in assays conducted over a range of 0.001–0.005 U of added enzyme activity. In these studies both exogenous phosphoryl choline labeled at the 2 position with [<sup>14</sup>C]oleate, and an endogenous phospholipid mixture labeled with [<sup>14</sup>C]AA and extracted from the microsomal fraction, were used in micellar suspensions as substrates for exogenous phospholipase  $\text{A}_2$ .

To further assess the specificity of inhibition of microsomal AH activity, effects of urea on microsomal PG synthetase were examined. In the absence of urea the microsomal fraction converted ~28–35% of the added AA (5 μM) to  $\text{PGE}_2$ ,  $\text{PGF}_{2\alpha}$ , or  $\text{PGD}_2$  ( $n = 3$ ). The relative distribution of the <sup>14</sup>C label among these moieties was 45–50%  $\text{PGE}_2$ , 26–34%  $\text{PGF}_{2\alpha}$ , and 20–

23%  $\text{PGD}_2$ . Inclusion of 1,000 mosmol/liter urea in the reaction mixtures did not alter the absolute conversion of exogenous AA to PG or the relative distribution of <sup>14</sup>C among the PG formed. By contrast, 100 μM indomethacin suppressed by >90% AA conversion to PG by the same microsomal preparations.

To assess the possible involvement of CaM in  $\text{Ca}^{2+}$ -responsive inner medullary AH activity, the effects of TFP were examined. In the presence of 100 μM  $\text{Ca}^{2+}$  in the enzyme assay mixture, 25 μM TFP significantly suppressed AH activity of both preparation I (basal 119±22 dpm/min per mg protein; +TFP, 67±9) and preparation II (113±18; +TFP, 47±5;  $n = 6$ ,  $P < 0.01$ ). TFP (25 μM) produced quantitatively similar suppression in the presence of 5 mM  $\text{Ca}^{2+}$ . By contrast, 25–100 μM PMZ or 100 μM indomethacin were without effect on microsomal AH activities of preparations I or II. Conversely, microsomal PG synthetase activity was markedly suppressed by 100 μM indomethacin but not by 25–100 μM TFP (not shown).

Table V shows the effects of  $\text{Ca}^{2+}$  and purified exogenous CaM on AH activity of an untreated 100,000 g par-

ticulate preparation (I). Particulate preparation II was washed with 10 mM EGTA at 4°C as described in Table IV. Particulate preparation III was washed with 10 mM EGTA at 4°C and subsequently incubated with 10 mM EGTA at 37°C for 20 min. Neither  $\text{Ca}^{2+}$  nor 1  $\mu\text{M}$  CaM altered AH activity of preparation I. In the absence of added  $\text{Ca}^{2+}$ , AH activity of preparation II was significantly lower than that of I, and was clearly increased by addition of  $\text{Ca}^{2+}$ . Addition of 1  $\mu\text{M}$  exogenous CaM however, did not alter AH of preparation II in either the presence or absence of  $\text{Ca}^{2+}$ . In the absence of  $\text{Ca}^{2+}$  or CaM, AH activity of preparation III was similar to the corresponding value for preparation II. The increase in AH observed in response to added  $\text{Ca}^{2+}$  however, was clearly reduced in preparation III compared with preparation II. Addition of 1  $\mu\text{M}$  exogenous CaM to preparation III restored  $\text{Ca}^{2+}$ -induced increases in AH to values comparable to those in preparation II. CaM had no effect on AH activity of preparation III in the absence of  $\text{Ca}^{2+}$ . As is also shown in Table V, the endogenous CaM-like activity of preparations I and II were similar, and

TABLE IV  
Responses to  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  of AH Activity in Inner Medullary Particulate Fractions Washed with EGTA

Additions	AH activity	
	I. Untreated fraction	II. EGTA-washed fraction
	dpm/min/mg protein	
None	97 ± 9	31 ± 4*
$\text{Ca}^{2+}$		
25 $\mu\text{M}$	89 ± 9	56 ± 6*†
100 $\mu\text{M}$	108 ± 12	94 ± 10†
5 mM	117 ± 14	106 ± 13†
$\text{Mg}^{2+}$		
5 mM	92 ± 10	37 ± 4*

Inner medullary slices (100 mg/2 ml) were incubated with [ $^{14}\text{C}$ ]AA (0.5  $\mu\text{Ci}/2$  ml) in complete KRBG for 2 h and washed with KRBG containing 5 mg/ml of fatty acid free albumin for 20 min. Slices were homogenized at 4°C in 50 mM Tris, pH 7.4, 5 mg/ml albumin (I) or the same buffer plus 10 mM EGTA (II). The 100,000 g particulate fraction was isolated and washed once at 4°C in Tris buffer of the same composition ( $\pm 10$  mM EGTA). Particulate fractions were resuspended in either 50 mM glycine, pH 9.0 (I), or 50 mM glycine, 10 mM EGTA (II). Enzyme assays were conducted in the presence (II) or absence (I) of EGTA, and contained, where indicated, either  $\text{Ca}^{2+}$ , final concentration 25  $\mu\text{M}$ –5 mM in excess of EGTA, or 5 mM  $\text{Mg}^{2+}$ . Results are means  $\pm$  SE of determinations pooled from four separate experiments ( $n = 8$ ).

\*  $P < 0.01$  compared to untreated fraction.

†  $P < 0.01$  compared to value in same fraction without added  $\text{Ca}^{2+}$ .

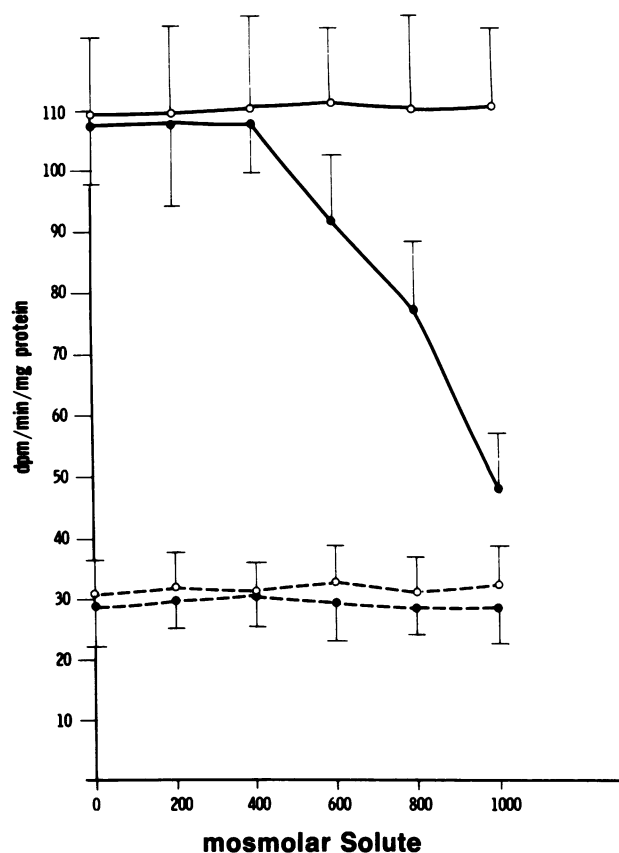


FIGURE 1 Inner medullary homogenates were prepared in 50 mM Tris, 10 mM EGTA, pH 7.4, 5 mg/ml albumin, and a washed 100,000 g particulate fraction isolated (preparation II, Table VI). The particulate fraction was resuspended in 50 mM glycine, 10 mM EGTA, pH 9.0, and incubated for 20 min at 37°C in the presence (O—O, ●—●) or absence (O---O, ●---●) of  $\text{Ca}^{2+}$  (100  $\mu\text{M}$  in excess of EGTA). Some incubations contained urea (●---●, ●—●), or NaCl (O---O, O—O), at the final concentrations indicated in the figure. At the end of the incubation the free AA content of the incubate was extracted, isolated by thin-layer chromatography, and counted as described in Methods. Results shown are means  $\pm$  SE of determinations pooled from three separate studies.

significantly greater than that of preparation III. Endogenous CaM-like activity was still readily detectable in preparation III but reduced to  $\sim 65\%$  of the content found in preparations I or II. Addition of 1  $\mu\text{M}$  exogenous CaM increased the CaM-like activity of the assay mixture nearly hundredfold to a value of 50  $\mu\text{g}/\text{mg}$  protein.

To assess the specificity of CaM action to restore  $\text{Ca}^{2+}$ -responsive AH activity of microsomal preparation III, effects of addition of other small acidic proteins were examined. At a concentration of 1  $\mu\text{M}$ , CaM restored  $\text{Ca}^{2+}$ -responsiveness of this preparation (basal,  $56 \pm 8$  dpm/min per mg protein; +CaM,  $123 \pm 6$ ),

**TABLE V**  
*Relationship between the Action of Exogenous CaM on AH Activity and Endogenous CaM-like Activity in the 100,000 g Particulate Fraction of Inner Medullary Homogenates*

100,000 g particulate fractions	AH activity				Endogenous CaM-like activity
	-CaM		+CaM		
	-Ca <sup>2+</sup>	+Ca <sup>2+</sup>	-Ca <sup>2+</sup>	+Ca <sup>2+</sup>	
	dpm/min/mg protein				
I. Untreated	115±9	112±14	107±11	110±12	0.74±0.12
II. EGTA-washed	38±5*	116±15‡	43±6*	102±11‡	0.72±0.09
III. EGTA-extracted	35±6*	58±9*‡	37±6*	118±14‡§	0.43±0.07*

Particulate fractions I and II were prepared as described in Table V. After washing at 4°C with 10 mM EGTA, particulate fraction III was also incubated for 20 min at 37°C in 50 mM Tris, 10 mM EGTA, 5 mg/ml albumin. This fraction was centrifuged at 100,000 g at 4°C. Particulate fractions were resuspended in either 50 mM glycine, pH 9.0 (I) or 50 mM glycine, 10 mM EGTA, pH 9.0 (II and III) for assay. Enzyme assays were conducted in the presence (II and III) or absence (I) of 10 mM EGTA and contained Ca<sup>2+</sup>, where indicated, at a final concentration of 100  $\mu$ M in excess of EGTA. Where shown, purified CaM was present at a final concentration of 1  $\mu$ M. CaM-like activity in the heat-treated (95°C min) 100,000 g particulate fractions was assessed by the ability of the preparations to stimulate activator-deficient phosphodiesterase activity as described in the Methods. Results shown are means  $\pm$ SE of determinations pooled from four separate experiments ( $n = 8$ ).

\*  $P < 0.01$  compared to corresponding activity in the untreated particulate fraction.

†  $P < 0.01$  compared to corresponding value in the absence of Ca<sup>2+</sup>.

§  $P < 0.01$  compared to corresponding value in the absence of CaM.

whereas addition of troponin C, ribonuclease, or cytochrome *c* was without significant effect.

## DISCUSSION

Results of the present studies demonstrate that the actions of hypertonic NaCl and mannitol to increase iPGE accumulation in inner medullary incubates (1) correlate with the stimulatory actions of these same solutes on the release of labeled AA from tissue lipid stores (Table I). Effects of NaCl and mannitol on these parameters are in striking contrast to those of hypertonic urea. Urea suppresses increases in [<sup>14</sup>C]AA release and iPGE, induced by either Ca<sup>2+</sup> plus A23187, or by hyperosmolar NaCl and mannitol. The increases in iPGE accumulation induced by NaCl or mannitol are not additive with those induced by exogenous AA. Moreover, the action of exogenous AA to increase iPGE is expressed in the presence of hyperosmolar urea (1). These findings are consistent with the proposal that NaCl and mannitol stimulate PGE generation by increasing the availability of free endogenous AA, a step that is rate limiting to cellular PG synthesis (4). Conversely, urea may suppress the stimulation of PGE accumulation mediated by A23187, NaCl, or mannitol by blocking actions of these agents to increase the availability of endogenous AA.

In contrast to the stimulation of [<sup>14</sup>C]AA release, and iPGE accumulation induced by A23187, which re-

quired the presence of Ca<sup>2+</sup> in the incubation media, the actions of NaCl or mannitol on these parameters are expressed equally well in either the presence or absence of Ca<sup>2+</sup> in the incubation media. Nevertheless, increases in these parameters induced by NaCl or mannitol were not additive with those induced by Ca<sup>2+</sup> plus A23187. Although other explanations are not excluded, stimulation of endogenous AA release by A23187, NaCl or mannitol through a final common pathway might account for these findings. It has previously been suggested that an increase in cytosolic Ca<sup>2+</sup> may mediate stimulation of renal medullary PGE synthesis induced by hyperosmolality or Ca<sup>2+</sup> ionophores (2, 7). This suggestion is in accord with (a) the presence of Ca<sup>2+</sup>-dependent pathways for the stimulation of AA release and PG synthesis in renal medulla and other tissues (1-3, 21), (b) the Ca<sup>2+</sup>-dependence of AH activity in vitro (4, 13, 22-24), and (c) studies from amphibian bladder and other tissues indicating that hyperosmolality may alter cellular Ca<sup>2+</sup> homeostasis (25-28). Observations from renal IM studies indicate that hyperosmolality stimulates Ca<sup>2+</sup> efflux (29). Thus, mobilization of cellular Ca<sup>2+</sup> could represent the pathway by which hypertonic NaCl or mannitol, as well as A23187, enhance AA release and PGE synthesis. Alternatively, the solutes, or A23187, might alter the structural arrangements of phospholipid membranes and thereby the susceptibility of the



lipids to cleavage by  $\text{Ca}^{2+}$ -dependent AH. This possibility has been suggested as a general mechanism for the stimulation of PG synthesis induced in intact tissues by various mechanical stresses such as distention, contraction, or vibration (4). Accordingly, the precise role of  $\text{Ca}^{2+}$  vs. structural changes in membranes, induced directly in intact cells by hypertonicity, in the expression of the actions of solutes on AA release and PG synthesis in IM, remains uncertain. However, because hypertonic urea, NaCl, and mannitol all stimulate calcium efflux in this tissue (29), it is unlikely that the inhibitory effect of urea on AA release and PGE synthesis is a simple function of calcium availability.

Several observations strongly imply that suppression of inner medullary PGE synthesis by hypertonic urea is related to its ability to inhibit  $\text{Ca}^{2+}$ -responsive AH activity. Studies with the 100,000 *g* particulate fraction provide, to our knowledge, the first evidence for the existence of  $\text{Ca}^{2+}$ -dependent particulate AH activity in renal IM (Table IV). Qualitatively similar  $\text{Ca}^{2+}$ -dependent AH activity has also been identified in examinations of plasma membrane fractions prepared from IM (20). These findings are consistent with studies in other tissues which have also demonstrated  $\text{Ca}^{2+}$ -dependent AH activity associated with microsomes and plasma membranes (4, 13, 22–24). Addition of hypertonic urea to the enzyme assay mixture, suppressed  $\text{Ca}^{2+}$ -responsive particulate AH of IM (Fig. 1), whereas hypertonic NaCl and mannitol were without effect. Analogous to urea, however, guanidine hydrochloride (1,000 mosmol/liter also inhibited particulate AH activity. While this agent shares with urea the capacity for protein denaturation and detergent action (30), it is unlikely that urea inhibition of AH is a simple function of these properties. Thus, 1,000 mosmol/liter urea failed to alter soluble phospholipase  $\text{A}_2$  activity or the content and distribution of [ $^{14}\text{C}$ ]AA among the lipid fractions of the microsomal preparations. Moreover, inhibition of microsomal AH activity by urea was highly selective compared with its effects on microsomal PG synthetase activity, which was unaffected by hypertonic urea. By contrast, indomethacin markedly suppressed PG synthetase but did not alter AH activity of the microsomal fraction. Thus, effects of these two agents on microsomal enzyme activities correlated well with their respective actions in the slice model. In the latter, urea suppressed both iPGE and [ $^{14}\text{C}$ ]AA release. Urea inhibition of iPGE was overcome by exogenous AA (1). Conversely, indomethacin inhibition of iPGE synthesis in the slice was not associated with changes in [ $^{14}\text{C}$ ]AA release, and was not overcome by exogenous AA (1). The selective inhibition, by urea, compared with NaCl or mannitol, of AA release and iPGE synthesis in an intact cell preparation of IM also correlates with selective inhibition by urea

of  $\text{Ca}^{2+}$ -responsive AH activity in the particulate fraction. This differential action provides a plausible basis for the selective inhibition of iPGE synthesis by hypertonic urea. The ability of hypertonic NaCl or mannitol to stimulate [ $^{14}\text{C}$ ]AA release and iPGE synthesis in an intact cell preparation contrasts with the absence of demonstrable stimulatory actions of these solutes on particulate AH activity (Fig. 2). The failure of NaCl and mannitol to augment AH activity upon direct addition to a broken cell preparation is in agreement with earlier observations (31). This finding may indicate that the effects of hypertonic NaCl and mannitol on AA release in intact cells are mediated indirectly through  $\text{Ca}^{2+}$ . As noted above, however, it is also quite possible that in the intact cell urea, NaCl, and mannitol alter differentially the structural arrangements of phospholipids in the membrane, and thereby increase or decrease lipid susceptibility to cleavage by  $\text{Ca}^{2+}$ -dependent AH. In this regard, the present results do not exclude the contingency that inhibitory actions of hypertonic urea and guanidine hydrochloride on AH reflect changes induced in the access of the particulate enzyme to lipid substrates within the membrane, rather than interference with  $\text{Ca}^{2+}$  interaction with the enzyme. Both urea and guanidine hydrochloride are known to disrupt hydrogen bonds and thus could alter the structural configuration of membrane bound AH, phospholipids, or both. This possibility requires further study.

The  $\text{Ca}^{2+}$ -binding protein, CaM, has been implicated in the expression of  $\text{Ca}^{2+}$  actions on numerous enzymatic reactions and biologic processes as an intracellular  $\text{Ca}^{2+}$  acceptor (9). Interaction of the  $\text{Ca}^{2+}$ -CaM complex with specific enzymes results in the conversion of the enzyme from a relatively inactive to a relatively active form. TFP, as well as other antipsychotic phenothiazines, are known to bind to the  $\text{Ca}^{2+}$ -CaM complex and prevent its interaction with target enzymes (32). Accordingly, inhibition by TFP of increases in [ $^{14}\text{C}$ ]AA release and iPGE accumulation induced in slices by  $\text{Ca}^{2+}$  plus A23187, or by hypertonic NaCl and mannitol, provide indirect evidence for the involvement of the  $\text{Ca}^{2+}$ -CaM complex in the activation of these processes in IM. By contrast, PMZ, a phenothiazine which binds poorly to the  $\text{Ca}^{2+}$ -CaM complex (32), was without effect on [ $^{14}\text{C}$ ]AA release or iPGE accumulation in inner medullary slice incubates or on particulate AH activity. Reduction in  $\text{Ca}^{2+}$ -responsive particulate AH by partial depletion of endogenous CaM-like activity provides further support for involvement of this  $\text{Ca}^{2+}$ -binding protein in the control of inner medullary AH. A role for CaM in the control of  $\text{Ca}^{2+}$ -responsive AH had been suggested from earlier studies of platelets (10). The difficulty in depleting endogenous CaM-like activity of the inner medullary particulate fraction implies its tight binding

in this preparation. This has also been observed in the particulate fraction of guinea pig brain (33). Even in brain however, the extent of CaM-like activity depletion achieved was greater than in IM (33). These findings could reflect the existence of different functional pools of CaM-like activity with variable membrane binding.

Regulation of a rate limiting step in PG synthesis by the  $\text{Ca}^{2+}$ -CaM complex may represent a pathway by which hormones, solutes, and other agents capable of altering cellular  $\text{Ca}^{2+}$  homeostasis influence PG generation. Whether urea inhibition of AH reflects interference with  $\text{Ca}^{2+}$  binding to CaM, alteration in the action of  $\text{Ca}^{2+}$ -CaM complex on AH, or primary structural changes in the membrane that occur independent of  $\text{Ca}^{2+}$  or CaM, remains to be determined. Moreover, the changes in free AA appearance observed in the present study of both the slices and particulate fractions, may reflect alterations in lipid reacylation, as well as deacylation. The former process was not monitored and could represent a step that is rate limiting with respect to the availability of free AA for conversion to prostaglandins. Whatever the precise mechanism, urea inhibition of AA release and PG synthesis in IM may subserve important homeostatic functions. To the extent that enhanced renal medullary PG generation tends to antagonize the hydroosmotic actions of vasopressin, as suggested by numerous observations (34–36), a high medullary urea concentration during hydropenia might potentiate, indirectly or directly, vasopressin action. Thus, in addition to the direct effect of a high interstitial urea concentration on passive water reabsorption (37), vasopressin action may be enhanced through the indirect effects of this solute to suppress inner medullary PG synthesis during hydropenia. Indeed, studies in our laboratory have now demonstrated that hypertonic urea inhibits the increases in iPGE synthesis induced in IM by either hypertonic NaCl or vasopressin (20). Thus, any action of NaCl to enhance PG synthesis in IM during hydropenia may be obviated by the concurrent rise in interstitial urea concentration. By contrast, urea does not suppress, and may potentiate the increases in cAMP and cAMP-dependent protein kinase activity induced by vasopressin (6). Accordingly, the high urea concentrations that pertain in IM during hydropenia permit expression of the biochemical events thought to mediate the antidiuretic activity of vasopressin (6), and may concurrently suppress local synthesis of PG antagonists of vasopressin action. Conversely, suppression of the hydroosmotic action of vasopressin under conditions of reduced medullary urea concentration may be due in part to enhanced local PG generation in these circumstances (1, 6, 38–42). However, because relatively high concentrations of urea were required to alter AA release and PGE synthesis, and be-

cause guanidine had effects on particulate acyl hydrolase activity qualitatively similar to those of urea, the importance of local alterations in urea concentrations in the physiologic regulation of inner medullary PG synthesis and water excretion remains to be determined.

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