Effects of Calcium on Vasopressin-mediated Cyclic Adenosine Monophosphate Formation in Cultured Rat Inner Medullary Collecting Tubule Cells

Evidence for the Role of Intracellular Calcium

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Abstract

We explored the effects of alterations in extracellular and intracellular calcium concentration on arginine vasopressin (AVP)-stimulated cAMP formation in cultured rat inner medullary collecting tubule cells. cAMP formation remains constant at extracellular calcium concentrations between 0.5 and 4.0 mM, which did not change intracellular calcium. Maneuvers that alter intracellular calcium concentration are associated with marked changes in cAMP generation. EGTA decreases intracellular calcium and enhances AVP-stimulated cAMP formation, while increasing cellular calcium with 2 μM A23187 decreases AVP-stimulated cAMP formation in the presence, but not in the absence, of extracellular calcium. The changes in cAMP formation observed when intracellular calcium is altered are associated with reciprocal changes in prostaglandin E_2 (PGE_2) synthesis. Despite >95% inhibition of PGE_2 synthesis with 5 μM meclofenamic acid, the changes in cAMP formation accompanying alterations in intracellular calcium concentration are still evident. These studies suggest that intracellular calcium critically influences AVP-stimulated cAMP formation. It does so by a mechanism independent of PG that is probably mediated by a direct effect of the cation on the adenylate cyclase complex.

Introduction

The association of hypercalcemia with polyuria, polydipsia, and a renal concentrating defect is well recognized both in man (1, 2) and in experimental animals (3, 4). The defect is clearly renal in origin, as vasopressin (AVP)[1] release is not impaired in hypercalcemia (5) and prevention of polydipsia does not prevent the development of a concentrating defect (4). The nature of the renal defect has been the subject of considerable investigation. Direct measurements of tissue solutes have shown that during hypercalcemia the concentration of solutes in the renal medulla is decreased (4, 6, 7). However, the observation that some hypercalcemic subjects elaborate urine that is hypotonic to plasma even in the presence of AVP (2, 3) strongly suggests an additional defect in the water permeability of the collecting duct. Studies in the toad urinary bladder (8, 9) and the perfused renal papilla (10) at very high (10 mM) concentrations of serosal calcium have demonstrated a decrease in the hydroosmotic response to AVP. In the isolated perfused cortical collecting tubule, however, a more modest (3.75 mM) elevation in peritubular (bath) calcium has been reported to enhance the hydroosmotic response to AVP (11).

The establishment of a link between the toad bladder studies and an effect of increased extracellular calcium to inhibit adenylate cyclase has been difficult. Thus, while only very high concentrations of the cation inhibit water movement, an increase from 0.1 to 0.5 mM calcium causes profound inhibition in adenylate cyclase activity in the toad bladder (12), inner medullary slices (13), and isolated medullary collecting ducts (14). In medullary slices, an increase to 2 mM calcium causes no further decrement in adenylate cyclase (13). The discrepancy between water flow and biochemical studies may well be due to the fact that the activity of the enzyme needs to be determined in broken cell preparations rather than in intact cells.

Another approach to the elucidation of the role of calcium in AVP action has involved the use of agents that either decrease calcium uptake by the cell (verapamil, nifedipine) or increase it (calcium ionophore A23187). However, the results with these agents have been inconsistent. Though verapamil has been reported to decrease the hydroosmotic response to AVP (15), this effect appears to occur by a mechanism other than calcium channel blockade (16). Taylor et al. (17) have reported that the calcium ionophore A23187 both inhibits and enhances (18) AVP-stimulated water transport.

Calcium also stimulates prostaglandin (PG) production in medullary slices of the rat kidney (19) and increases urinary PG excretion in both the anesthetized dog (20) and the conscious rat (4). As PG are known to antagonize the hydroosmotic response to AVP (21, 22), it is attractive to postulate that the concentrating defect in hypercalcemia is mediated by PG. PG inhibition has been reported to attenuate the concentrating defect of hypercalcemia in one study (23), but it failed to do so in two others (4, 24). However, PG inhibition in hypercalcemia is associated with significant decreases in renal blood flow and glomerular filtration rate (25); this could obscure an improvement in urinary concentrating ability.

The present studies were undertaken to further explore the mechanism of the altered hydroosmotic response to AVP observed in hypercalcemia by studying the effects of calcium in an intact cell system. We sought answers to the following questions: (*a*) Does calcium affect AVP-mediated cAMP production in such a system? (*b*) What are the relative roles of extracellular and intracellular calcium in the process? (*c*) Are the effects of calcium mediated by the PG system?

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1. *Abbreviations used in this paper:* cAMP, cyclic AMP; AVP, arginine vasopressin; FBS, fetal bovine serum; PG, prostaglandin; PGE_2, prostaglandin E_2; SDS, sodium dodecyl sulfate.

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Methods

Inner medullary collecting tubule cell culture. Tissue was prepared by a modification of the method of Grenier et al. (26). Specifically, male Sprague-Dawley rats were exsanguinated, and their kidneys were immediately removed using sterile technique and placed in ice-cold Krebs-Ringer's buffer (composition in mM: NaCl, 128, KCl, 5, $CaCl_2$, 1.0, $MgSO_4$, 1.2, Na CH_3COO, 10, NaH_2PO_4, 2.0, glucose, 10, and Tris, 10), pH 7.4, containing 100 U/ml penicillin and 100 μg/ml streptomycin base. The kidneys were then bisected and inner medullae from 4 to 12 rats pooled, finely minced with a sterile razor blade, and incubated in 5 ml of 0.05–0.1% collagenase (Worthington Biochemical Corp., Freehold, NJ) in Krebs-Ringer's buffer for 60–90 min at 37°C under a 5% CO_2 atmosphere. The tissue was gently agitated at 15–20-min intervals. After incubation, 2 vol sterile distilled water was added to lyse cells other than collecting tubule cells. The tissue was pelleted by centrifugation for 3 min in a tabletop clinical centrifuge, resuspended in 10% bovine serum albumin (fraction V; Miles Laboratories Inc., Elkhart, IN), and centrifuged again. The remaining cell pellet was resuspended in 90% Ham F-12–10% Liebovitz L-15 medium containing 10% fetal bovine serum (FBS), 1% antibiotic, and hormonal supplements (27). Tissue was seeded into 24-well (16 mm diameter) replicate culture dishes (Falcon Labware, Div. of Becton-Dickinson & Co., Oxnard, CA). The seeding density was such that ~12 wells were seeded per rat initially killed. The cells were then kept under a 5% CO_2 atmosphere at 37°C. After 24 h the cells were fed with the same medium that contained, however, only 1% FBS. Experiments were performed at 72–96 h in a nonconfluent state, and at a time when each well contained 30–60 μg of protein.

Incubation of cells with effectors for cAMP determination studies. Studies were performed in 24-well culture dishes. Media was aspirated off the cells and replaced with 0.5 ml Krebs-Ringer's buffer, pH 7.4, which contained the desired effector substance. In all cases, any given effector was studied in 3–4 wells providing either a triplicate or quadruplicate determination. All studies were performed at 300 mosmol/kg H_2O and in the presence of 0.2 mM methyl-isobutyl xanthine (Calbiochem-Behring Corp., La Jolla, CA) to inhibit phosphodiesterase. Incubations were done in a Fisher Isotemp dry bath at 37°C. As intracellular cAMP has been demonstrated to peak after a 5–10-min exposure to AVP (26), all incubations for cAMP measurements were performed for 7.2 min. At the end of this time the incubation was terminated by aspiration of the effector and the immediate addition of 0.3 ml of 0.01 N HCl to lyse the cells and liberate cellular cAMP. After 20 min, a 100-μl aliquot of acid was taken and immediately frozen for subsequent determination of cAMP content. cAMP was determined by radioimmunoassay (New England Nuclear, Boston, MA) with standards similarly dissolved in 0.01 N HCl. Recovery was 100.48±1.84% (n = 6). As treatment of the cells with HCl caused membrane fragmentation and loss of protein into the acid, a second 100-μl aliquot of the acid from each sample was taken for protein determination. The residual acid was aspirated off and the remaining tissue solubilized in 1% sodium dodecyl sulfate (SDS). A 100-μl aliquot of the SDS was added to the second 100-μl aliquot of HCl acid and the combined protein content was determined by the method of Lowry (28). Results were expressed as femtomoles cAMP per microgram protein with the mean of each triplicate or quadruplicate taken as an n of one.

Incubation of effectors for PG studies. In studies designed to assess PG production, the preparation of tissue and the composition of the buffer was the same, but the incubation time allowed was 10 min. Then, 100 μl of the incubation media was sampled and frozen for determination of PGE_2 content by radioimmunoassay (29) using an antibody supplied by the Pasteur Institute, Garches, France. Unknowns and standards are incubated with [^{3}H]PGE_2 (New England Nuclear) and free antigen is separated by adsorption onto charcoal-dextran and centrifugation. Intraassay variability is 6%, and interassay variability is 10%. Since no extraction step was required we found our recovery to be essentially 100%. After aspiration of the rest of the incubation medium, the protein content was determined by the Lowry method on the cells that remained at the bottom after exposure to SDS (28). PG production was expressed as picograms per microgram protein per 10 min. The triplicate or quadruplicate determinations in any given experiment were meaned for an n of one.

Measurement of intracellular calcium. To determine whether a given alteration in extracellular calcium concentration resulted in a change in the intracellular concentration of the cation, cytosolic-free Ca^{++} was measured by the use of the new highly fluorescent indicator fura-2 (30). Tissue was prepared as described above but then seeded at a higher density into 35-mm wells. Tissue was fed on days 1 and 2 as above and again on day 4 with media containing 1% FBS. At day 6, cells were removed from the plates and disaggregated by the use of 0.25% Trypsin-0.1% EDTA for 20 min at 37°C. Cells were then gently spun in a table top centrifuge, washed, respun, and resuspended in Krebs-Ringers buffer (as above). From this preparation, we obtained 1.9–4.2 × 10^6 cells/sample, which is more than sufficient for cytosolic Ca^{++} determinations. Viability was >95% as assessed by nigrosin dye exclusion. The cells were then loaded with fura-2AM (2 μM) for 20 min, fura-2AM was then diluted to 1 μM for an additional 20 min.

Excess fura-2AM was then removed by washing in a calcium-free buffer (without EGTA). The cells were then divided into three aliquots. In group 1, the cells were exposed to 0.33 mM $CaCl_2$ + 1 mM EGTA; this resulted in an extracellular calcium concentration of 0.7 μM. In groups 2 and 3, extracellular calcium was 1.0 and 4.0 mM, respectively. Over the ensuing 50 min, repeat measurements of fluorescence at 347 and 387 nm, which corresponded to the maximum and minimum calcium-dependent fluorescence, respectively, were obtained on a Turner model 430 spectrofluorimeter equipped with a stirring device and a 37°C circulating water bath. These wavelengths were determined after obtaining excitation spectra with fura-2 at various calcium concentrations. After correction for autofluorescence, the cytosolic Ca^{++} concentration was calculated from the equation:

$$[Ca^{++}] = K_d((R - R_{min})/(R_{max} - R))((S_{f2})/(S_{b2}))\ (30). \quad (1)$$

Dissociation constant (K_d) was 224 nM (30); R_{min}, R_{max}, and S_{f2}/S_{b2} were derived (Draznin, B., J. W. Leitner, M. L. Kao, and K. E. Sussman, submitted for publication) using the spectral data for fura-2 at zero (1 mM EGTA) and saturating (1 mM) calcium in both Krebs-Hepes buffer, pH 7.4, and in a buffer similar to that employed by Grynkiewicz et al. (30) that reflects the intracellular environment with a high potassium and low sodium concentration at an excitation wavelength of 347 nm (λ_1) and 387 nm (λ_2). With either buffer, the following values were obtained: R_{min} = 0.707, R_{max} = 5.442, and S_{f2}/S_{b2} = 3.684. R is the ratio of fluorescence at λ_1/λ_2, and S_{f2} and S_{b2} are the fluorescence intensities of fura-2 in the free and bound forms at λ_2. Emission was determined at 478 nm.

To ascertain whether the results obtained in the presence of EGTA were in part accounted for by a significant leak of fura-2 out of the cells, we employed manganese, which has an affinity for fura-2 42× greater than that of calcium (30). To ensure that this cation indeed displaces calcium, we determined the concentration of the calcium–fura-2 complex in a cell-free buffer and of 2 μM fura-2 (free acid) that contained 1.0 mM $CaCl_2$ before and after the addition of manganese in equimolar quantity. The calcium–fura-2 concentration was 1,646 nM when measured before manganese addition. This cation immediately decreased the concentration to 46 nM, thus demonstrating 97% displacement of calcium from the fluorescent marker. The magnitude of the fura-2 leak from our cells was determined in three studies in which the cells were prepared and loaded with fura-2 as described above. The cells were then divided into two aliquots, one of which was treated with EGTA and the other with 1 mM manganese. The intracellular calcium concentration was then measured frequently over a 4-min period.

Studies on the role of extracellular calcium. To assess the effect of changes in the concentration of calcium that bathes the cells on their ability to generate cAMP, dose-response curves to AVP (0, 10^{-9}, 10^{-8}, and 10^{-7} M) were generated as buffer calcium was altered. To minimize potential secondary changes in the cellular concentrations of calcium, initial studies were performed without preincubation, which thus exposed the cells to the desired calcium concentration for only the duration of

the 7.2-min experimental incubation. When compared with physiologic 1-mM calcium, the concentration was decreased by 50% to 0.5 mM and increased to 2.0 and 4.0 mM. To expose the cells to a high calcium concentration for a longer period, the cells were also preincubated with 4 mM calcium for as long as 60 min at 37°C under 5% CO_2, before the 7.2-min experimental incubation described above.

Studies on the role of intracellular calcium. To determine the effect of alterations in cellular calcium on AVP-mediated cAMP formation, dose-response curves to AVP (0–10^{-7} M) were generated in settings that either decrease or increase intracellular calcium. Intracellular calcium was decreased by incubation in calcium-free medium for 1 h (effective calcium concentration as measured by calcium-specific electrode [2.5 μM]) or by addition of 1 mM EGTA and 0.33 mM calcium (effective calcium concentration of 0.7 μM).

In addition, to better define the kinetics whereby the decrease in cellular calcium alters the cAMP response, we studied cAMP generation at four concentrations that approximate the K_m of the response (10^{-9}, 5×10^{-9}, 10^{-8}, 5×10^{-8} M AVP) at 1 mM calcium and after a 1-h preincubation in the virtual absence (2.5 μM) of calcium in the buffer. The resulting curves were subjected to Lineweaver-Burk transformation to determine whether the observed alteration in cAMP generation was due to a change in Michaelis-Menten constant (K_m) or maximum velocity (V_{max}).

The maneuver employed to increase the cellular content of calcium involved the use of the calcium ionophore A23187 (Calbiochem-Behring Corp.). Thus, AVP dose-response curves were generated in the presence and absence of the ionophore (2 μM). A calcium-independent effect of the agent to alter cAMP production was determined by studying its effect both in the absence of extracellular calcium (0.7 μM) and the presence of either 1 or 4 mM calcium. In all these studies directed at altering intracellular calcium, cells were preincubated at the desired calcium concentration with or without the test substance for 60 min. Concurrently, the buffer was replaced with the effector solution of identical composition and the response to AVP studied over the 7.2-min experimental incubations as discussed above.

Measurement of cellular ATP content. To ensure that any effect observed in the presence of ionophore is not due to a nonspecific toxic effect of this agent, cellular ATP content was determined in the absence and presence of the drug. Cells were preincubated in buffer at 1.0 mM calcium without or with A23187 as described above. Buffer was aspirated off and the cells transferred with a rubber policeman into tubes containing ice-cold 0.56 N perchloric acid. An aliquot was then removed for determination of protein (28). After a 20-min centrifugation at 3,000 rpm the perchloric acid was neutralized by the addition of 5 M K_2HPO_4 (15% by volume). The tissue was kept on ice for 15 min, spun again at 3,000 rpm for 20 min, filtered, and immediately frozen in liquid nitrogen. ATP content was subsequently determined by high performance liquid chromatography using a modification of the method of Schweinsberg et al. (31).

Studies on the role of PG

Measurement of PGE_2 production. To explore a possible role for PG in mediating the effect of changes in calcium we measured PGE_2 production in the presence of 10^{-7} M AVP at 0.7 μM and 1 mM extracellular calcium ($n = 8$) as well as in the presence of the calcium ionophore ($n = 5$). Cells were preincubated for 60 min as previously described. Buffer was aspirated and replaced by the desired effector solution. After 10 min an aliquot of the effector solution was taken for measurement of PGE_2 production.

Studies on the relationship between changes in PGE_2 and cAMP production. To determine whether the calcium-mediated changes in PGE_2 production are responsible for the alterations in cAMP generation, we used a cyclooxygenase inhibitor that would prevent PG synthesis and yet not directly affect the cAMP system. To this end, dose-response curves to AVP were obtained in the presence and absence of 5 μM meclofenamate (Warner-Lambert Co., Lititz, PA). This was done both in the setting of decreased (EGTA) and increased (ionophore) intracellular calcium.

Statistical analysis. Comparison between any two experimental settings was done with the unpaired t test. When comparisons involved multiple groups, an analysis of variance was used (32). All data are expressed as mean±SE. A P value of <0.05 was considered significant.

Results

Morphological and biochemical characterization of the inner medullary collecting tubule cell in culture. By 72 h after plating, a monolayer (nonconfluent) of cells is apparent (Fig. 1 *A*). These cells maintain their polarity in culture, with the basolateral membrane oriented toward the growth surface and the apical (luminal) membrane facing the media (Fig. 1 *B*). A homogeneous population of rat inner medullary collecting tubule cells is evident. These cells synthesize adenosine 3′,5′-cAMP in response to AVP in dose-dependent fashion (see below). Intracellular cAMP (femtomoles per microgram protein per 7.2 min) increased from 29.51±5.53 in the basal state to 210.26±18.62 at 10^{-6} M AVP. To ensure that the response to AVP was specific, the ability of a number of other substances to stimulate cAMP was tested (Fig. 2). Neither parathyroid hormone (0.5 U/ml), calcitonin (3×10^{-7} M), or glucagon (10^{-6} M) significantly increases cAMP above basal levels. Although the increase observed with isoproterenol (10^{-6} M) is significant ($P < 0.05$), it is far lower than that seen with 10^{-7} M AVP ($P < 0.001$). The overall pattern of hormonal response, with AVP-sensitive adenylate cyclase predominating, and only minimal response to other substances, is in keeping with that reported for individually dissected rat inner medullary collecting tubules (34).

Studies on the role of extracellular calcium (Figs. 3 and 4, Table I). To determine whether acute changes in the calcium concentration of the media bathing inner medullary collecting tubule cells alter the response to AVP, we compared physiologic calcium (1 mM) with a 50% decrease (0.5 mM) or a doubling of calcium concentration (2 mM). As is depicted in Fig. 3, neither the increase nor the decrease in the bathing concentration of the cation causes any significant change in this response. So as to mimic the maximal possible concentration of the cation that could bathe the inner medulla during severe hypercalcemia, we also tested the effect of 4 mM extracellular calcium and allowed for a 1-h preincubation. As seen in Table I, the cytosolic Ca^{++} concentration of 184.4±14.7 nM obtained in cells exposed to an extracellular calcium concentration of 4.0 mM is virtually identical to the 190.0±11.6 nM determined in cells bathed in 1.0 mM extracellular calcium. As is depicted in Fig. 4, although the cAMP response at 4.0 mM extracellular calcium was minimally lower than that at 1.0 mM extracellular calcium, it failed to reach statistical significance. Therefore, even profound alterations in the concentration of calcium bathing inner medullary collecting tubule cells cause no change in intracellular calcium concentration and have no effect upon the ability of these cells to generate the cellular mediator of AVP.

Studies on the role of intracellular calcium. In view of the above results we sought to determine whether maneuvers that alter the concentration of calcium within the cell are associated with changes in cAMP synthesis.

Effect of decreasing cellular calcium (Figs. 5, 6, and 7, Table I). To decrease cellular calcium we removed calcium from the bathing media or chelated it with 1 mM EGTA. As shown in Table I, the combination of 0.33 mM Ca + 1.0 mM EGTA (resulting in a free [Ca^{++}] of 0.7 μM) causes a dramatic decrease in cytosolic calcium from 190.0±11.6 nM to 64.8±4.6 nM, P

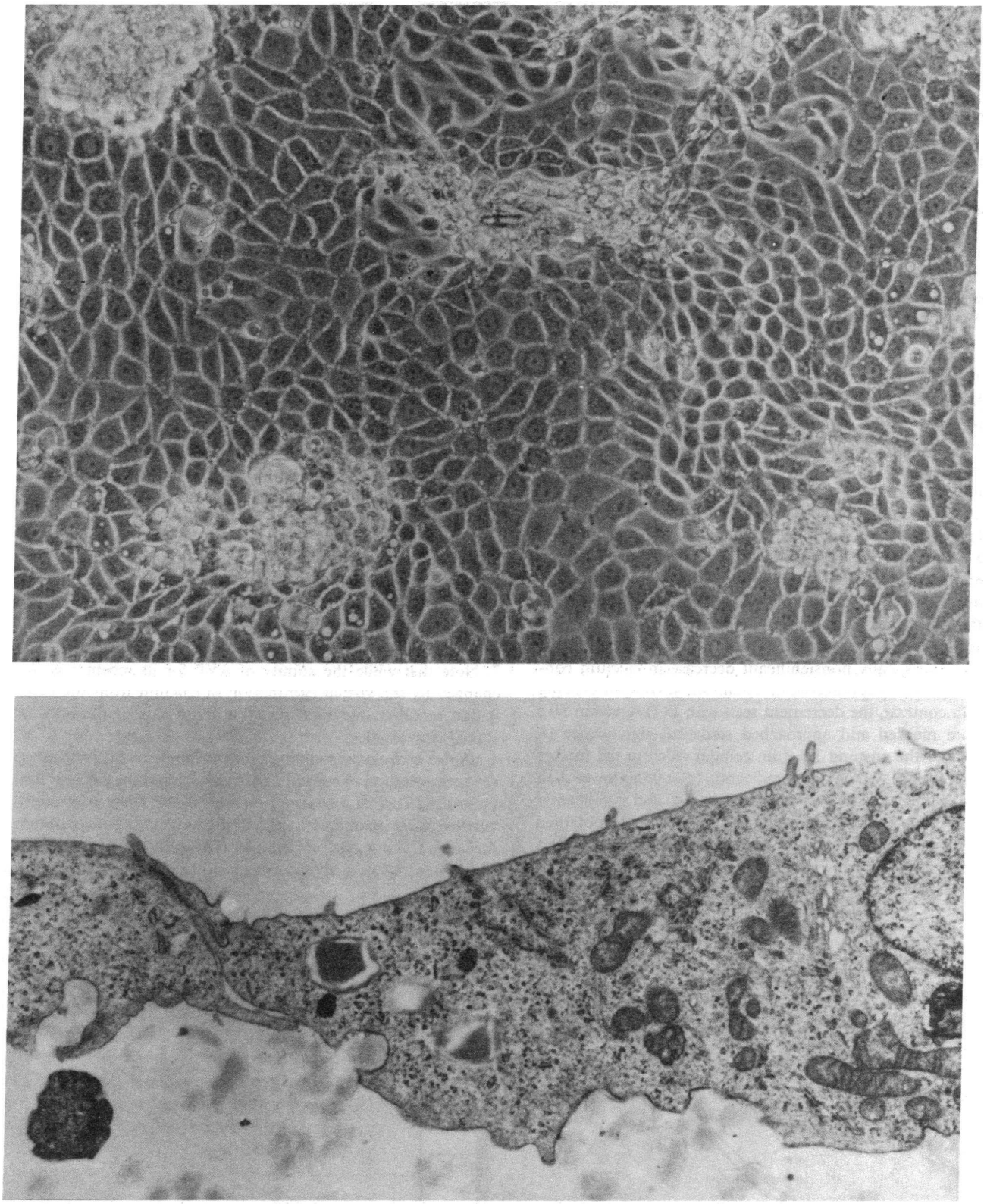

Figure 1. (*A*) Phase-contrast photomicrograph of cultured rat inner medullary collecting tubule cells. Original magnification × 200. (*B*) Electron micrograph × 13,100. Note the scattered apical microvilli and the well-developed tight junction.

< 0.001. This decrease was evident as early as 6 min after exposure to EGTA and remained stable for the ensuing 50 min. Fig. 5 demonstrates the enhanced cAMP response that we saw in this setting. The production of cAMP was consistently higher in the virtual absence of calcium in the media for 60 min ($P < 0.05$). A very similar enhancement is noted when cells are preincubated for 60 min in calcium-free buffer (0.0025 mM), thereby demonstrating that the enhancement is not due to EGTA

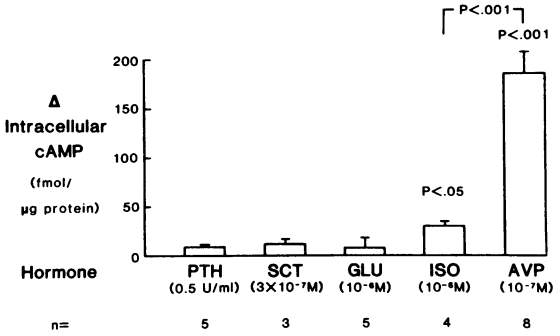

Figure 2. Pattern of hormonal response in cultured RIMCT cells. PTH, parathyroid hormone; SCT, salmon calcitonin; GLU, glucagon; and ISO, isoproterenol.

itself (data not shown). Likewise, we assessed the effect of EGTA on cAMP formation in response to 10^{-7} M AVP in suspended cells processed in the same manner as those used for the cellular calcium measurements. In six determinations obtained from two experiments, while the exposure to trypsin decreased the cellular response to AVP, the response was enhanced by EGTA (129.1±11.75 vs. 88.0±4.8 fmol/μg protein at 1.0 mM Ca^{2+}, $P < 0.01$).

To ensure that the decrease in measured cytosolic calcium that we observed with EGTA was not a consequence of a leak of fura-2 from cells, we used 1 mM manganese. As is evident from the results of three experiments using the same cell population (depicted in Fig. 6), manganese immediately caused a small but statistically nonsignificant decrease in calcium concentration, which was observed to remain stable over the ensuing 4 min. In contrast, the decrement seen with EGTA within 30 s was more marked and approached statistical significance ($P < 0.1$). Over the ensuing 3.5 min, cellular calcium fell further to 82.5±11.0 nM, which was significantly ($P < 0.05$) lower than the 30-s value of 103.5±13.5 nM (paired t test) and significantly ($P < 0.05$) different from the value of 126.2±8.1 nM obtained with manganese. These results clearly demonstrate that while a small leak of fura-2 from the cells may occur, the decrease in calcium-dependent fura-2 fluorescence observed upon addition

Table I. Effect of Alterations in Extracellular Calcium on the Concentration of Intracellular Calcium

Study	Extracellular calcium	0.33 mM + 1.0 mM EGTA (0.7 μM)	1.0 mM	4.0 mM
		Intracellular calcium (*nM*)		
1		69.9	157.8	170.3
2		72.1	212.4	153.0
3		65.3	199.4	220.6
4		51.8	190.5	193.7
	x̄±SE	64.8±4.6	190.0±11.6	184.4±14.7
	P value	<0.001		NS

of EGTA most likely represents a true decrease in cytosolic calcium concentration. The numbers presented in Table I have been adjusted to correct for this small leak.

We considered the possibility that these effects of EGTA were due to greater exposure of the hormone to its receptors on the basolateral membrane, in contact with the dish, as a consequence of changes in the tight junction. However, electronmicrographs of the cells exposed to the low-media calcium showed no evidence for such a disruption.

To better define whether the enhanced production of cAMP in this setting is due to an effect on the affinity of AVP to its receptor or on the maximal velocity of adenylate cyclase activity, we studied the response to the hormone at four concentrations of AVP that are close to the apparent K_m of 5×10^{-9} M. The Lineweaver-Burk transformation of this data is depicted in Fig. 7. Note that while the affinity of AVP for its receptor is unchanged by the virtual elimination of calcium from the incubation medium, maximal reaction velocity is significantly ($P < 0.02$) increased.

Effect of increasing intracellular calcium (Fig. 8). To increase the concentration of cytosolic calcium we used the calcium ionophore A23187 at a concentration of 2 μM. Cells were preincubated with ionophore at the desired calcium concentration (0.7 μM, 1 and 4 mM) for 60 min. The effects of ionophore on cAMP generation were dependent on the extracellular concen-

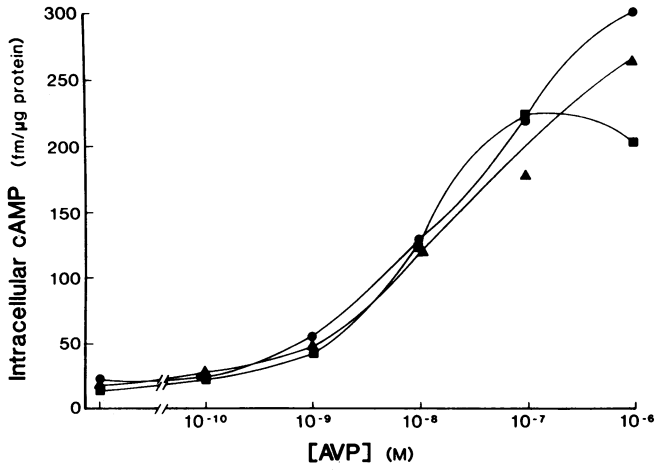

Figure 3. Dose-response curves to AVP at varying concentrations of extracellular calcium. Each point represents the mean of six determinations. (— ● —) 0.5 mM; (— ▲ —) 1.0 mM; and (— ■ —) 2.0 mM.

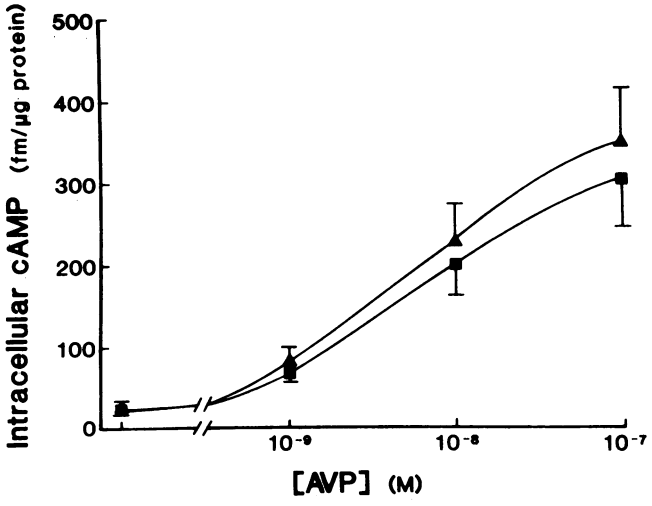

Figure 4. Dose-response curves to AVP after a 60-min preincubation at 1.0 mM (— ▲ —) and 4.0 mM (— ■ —) extracellular calcium. Each point represents the mean of six determinations.

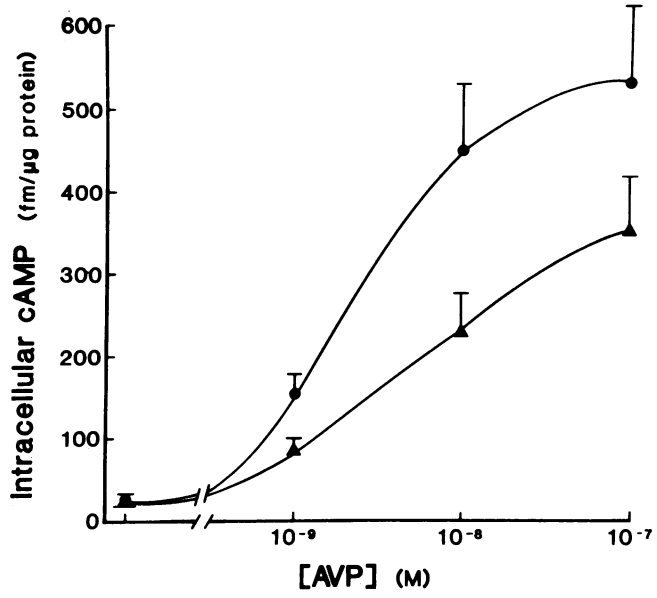

Figure 5. Dose-response curves to AVP after a 60-min preincubation at 1.0 mM Ca^{++} (— ▲ —) and after intracellular calcium depletion by EGTA (— ● —); buffer Ca^{++} 0.7 μM. Each point represents the mean of six determinations. The production of cAMP is consistently higher when cellular calcium is decreased ($P < 0.05$).

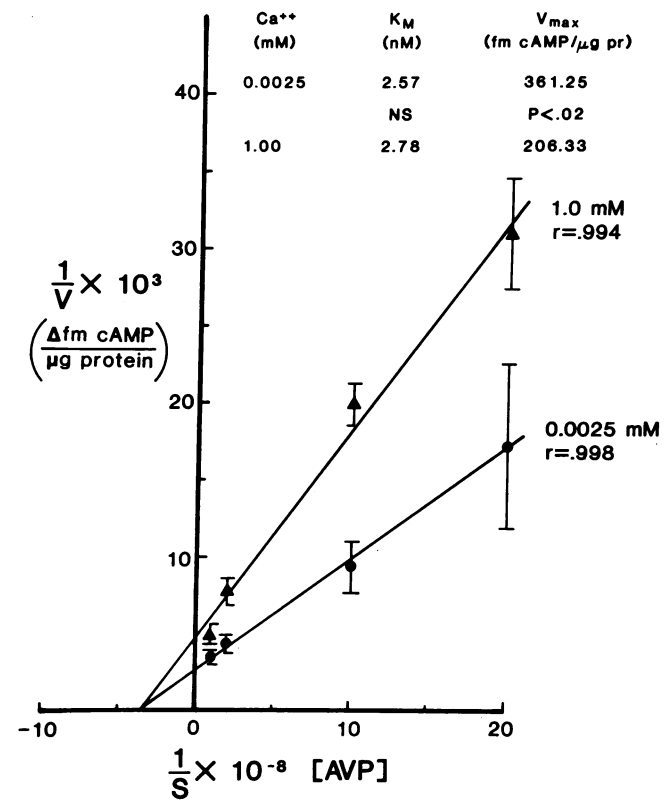

Figure 7. Lineweaver-Burk transformations of dose-response curves to AVP after a 60-min preincubation at 1.0 mM (— ▲ —) and 0.0025 mM (— ● —) Ca^{++}. Each point represents the mean of five determinations.

tration of calcium (Fig. 8). In the virtual absence of the cation (0.7 μM) ionophore did decrease cAMP formation, but this decrease was not statistically significant. In contrast, at 1 mM extracellular calcium the ionophore significantly ($P < 0.05$) decreased cAMP formation. The decrement averaged 57.6±3.6%, a decrease that is significantly greater than that seen at 0.7 μM, 37.2±3.2% ($P < 0.005$). Likewise, at 4 mM extracellular calcium the ionophore caused a significant decrease in cAMP formation ($P < 0.05$) and the percentage decrease 68.0±4.4% was significantly greater than that observed at 1 mM calcium ($P < 0.05$). Apparently a maneuver that certainly increases the cellular concentration of calcium profoundly inhibits the formation of the mediator of vasopressin's hydroosmotic effect.

Measurements of cellular ATP content (Table II). To ensure that the decrease in cAMP formation observed with ionophore

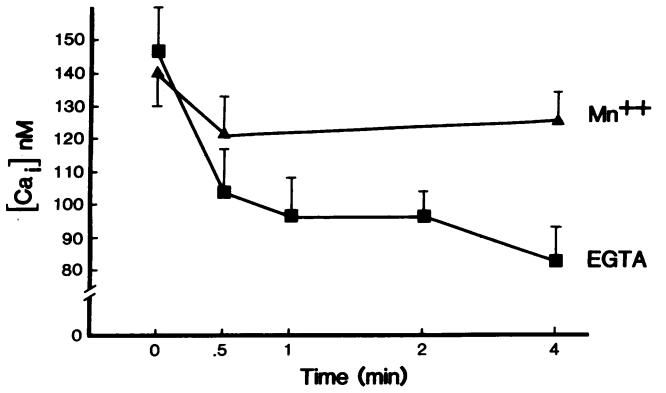

Figure 6. Effect of EGTA (1.0 mM + 0.33 mM Ca^{++}) and manganese (1.0 mM) on measured cellular calcium. Manganese (— ▲ —) causes a small, not significant decrement in the measurement, which is stable with time. The decrease noted with EGTA (— ■ —) is greater and is more pronounced with time ($P < 0.05$).

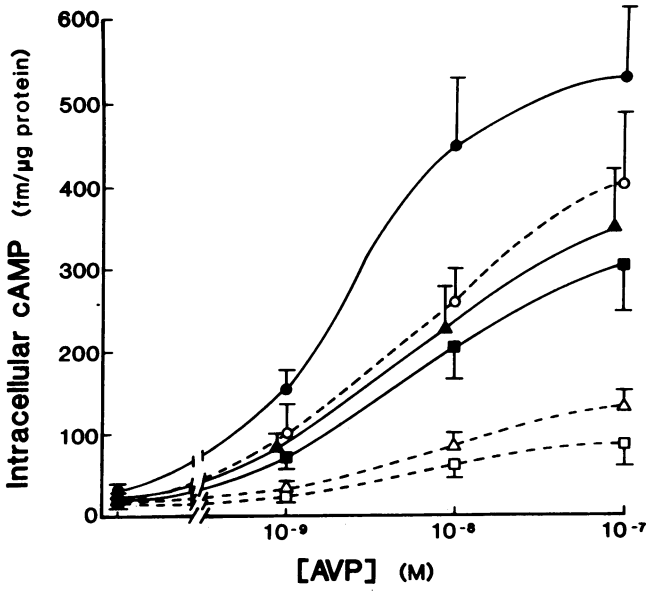

Figure 8. Dose-response curves to AVP after a 60-min preincubation at varying concentrations of calcium in the absence (——) and presence (-----) of the calcium ionophore A23187 (ionophore, 2 μM). Each point represents the mean of six determinations. (— ● —) 0.7 μM; (- - ○ - -) 0.7 μM + ionophore; (— ▲ —) 1.0 mM; (- - △ - -) 1.0 mM + ionophore; (— ■ —) 4.0 mM; (- - □ - -) 4.0 mM + ionophore.

Table II. Effect of 2 μM A23187 on Cellular ATP Levels

Experiment	ATP (pmol/μg protein) 1.0 mM Ca	1.0 mM Ca + A23187
1	22.60	16.05
2	15.60	17.23
3	15.83	13.37
x̄±SE	18.01±2.30	15.55±1.14
	NS	

is not due to a nonspecific toxic effect of the agent, we measured cellular ATP levels in cells incubated at 1.0 mM calcium without and with A23187. The results portrayed in Table II show that ATP content is comparable in the two groups. Further support for the absence of a toxic effect of the drug is derived from the absence of any visible electron microscopic changes (data not shown).

Studies on the role of PG in calcium-mediated changes in cAMP formation

Measurement of PGE_2 production (Fig. 9). Since PGE_2 is an important modulator of antidiuretic hormone action, we measured PGE_2 production by our cells in the settings in which cAMP was altered. As shown in Fig. 9, PG production is very low in the absence of calcium, while the calcium ionophore in the presence of extracellular calcium markedly stimulates PGE_2 synthesis ($P < 0.01$).

Studies with inhibition of PG synthesis (Fig. 10, Table III). The pattern of PG synthesis observed with maneuvers that increase or decrease cellular calcium raised the possibility that the calcium-induced changes in cAMP generation could be mediated by the reciprocal changes in PGE_2 synthesis. To explore this possibility we used the cyclooxygenase inhibitor meclofenamic acid (5 μM). Table III demonstrates that even in conditions tailored to maximize PGE_2 synthesis (buffer osmolality 1,200 mosmol per kg H_2O, 4 mM extracellular calcium + 2×10^{-6} M A23187), this dose of meclofenamic acid inhibited PGE_2 production by >95%. Thus, this dose of the cyclooxygenase inhibitor was used to ascertain whether the observed changes in cAMP production would be abolished in the setting of PGE_2 inhibition. Fig. 10 shows that the enhanced production of cAMP that we saw with EGTA and the marked inhibition associated with the use of ionophore at either 1 or 4 mM calcium are in no way altered by meclofenamic acid (compare with Fig. 8). It appears therefore that cyclooxygenase products are not the mediators of the observed effect.

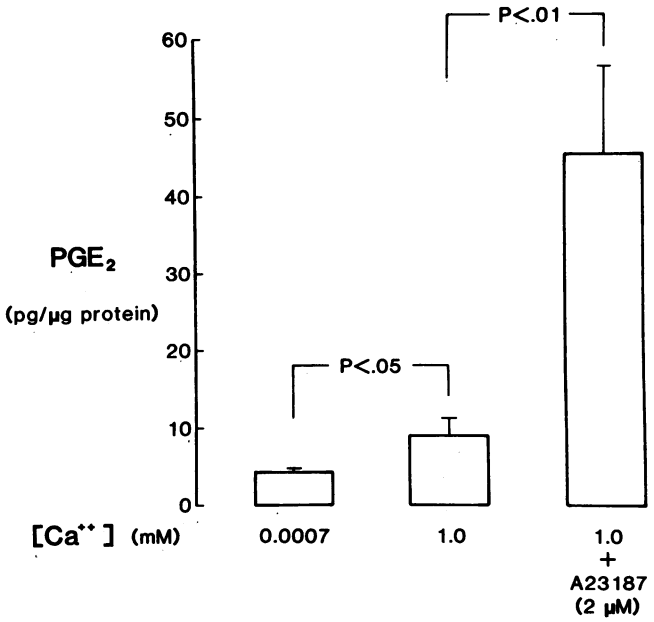

Figure 9. PGE_2 synthesis at varying concentrations of extracellular calcium. Values for 0.0007 mM and 1.0 mM represent the means of eight determinations, and for 1.0 mM + A23187 the mean of five determinations.

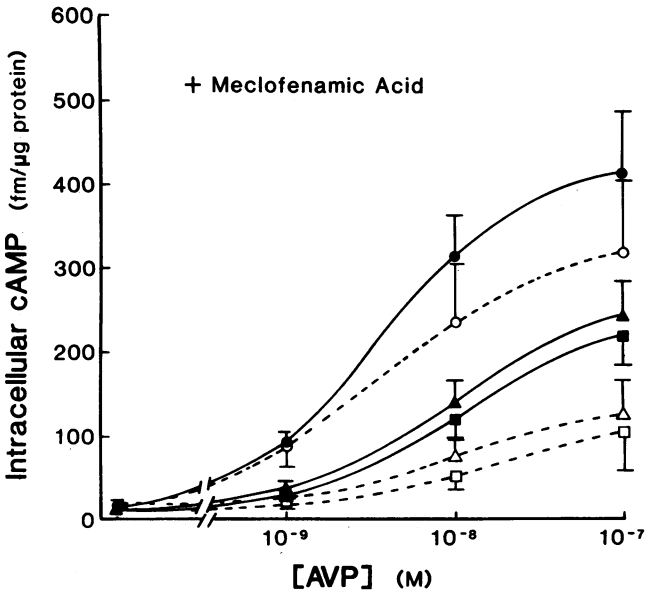

Figure 10. Dose-response curves to AVP after a 60-min preincubation with 5 μM meclofenamic acid at varying concentrations of calcium in the absence (——) and presence (-----) of the calcium ionophore A23187 (ionophore, 2 μM). Each point represents the mean of four determinations. (— ● —) 0.7 μM; (- - ○ - -) 0.7 μM + ionophore; (— ▲ —) 1.0 mM; (- - △ - -) 1.0 mM + ionophore; (— ■ —) 4.0 mM; and (- - □ - -) 4.0 mM + ionophore.

Discussion

The well-recognized disturbance in the renal concentrating process that occurs in hypercalcemia makes the understanding of the mechanism whereby this cation affects the action of AVP particularly relevant. While extremely high nonphysiologic levels of calcium (10 mM) applied to the serosal surface of the toad

Table III. Effect of 5 μM Meclofenamic Acid on Ionophore (A23187, 2×10^{-6} M)-stimulated PGE_2 Synthesis

Ca^{++}	Number	Ionophore	Ionophore + meclofenamate	Inhibition
mM		*pg/μg/h*	*pg/μg/h*	*%*
1.0	5	72.61±8.96	2.29±1.42	96.85
4.0	3	153.96±14.41	3.71±3.52	97.59

urinary bladder inhibit AVP-mediated water flux (8, 9) the activity of adenylate cyclase is clearly inhibited at concentrations below 1 mM (12). However, the physiologic significance of this observation is also questionable, as the enzyme assay is performed in membrane preparations whereas the catalytic unit of the enzyme complex is never exposed to this calcium concentration in the intact cell. Attempts to determine the effects of high calcium in in situ cells have yielded conflicting results in two preliminary studies that used dissected tubular segments. One of these has found a decrease in cAMP accumulation (14) while another found no such effect in isolated collecting ducts (34). The studies herein described are the first to use cultured inner medullary collecting tubule cells to methodically study whether and how perturbations in the extracellular and intracellular concentrations of calcium affect the in situ cAMP system in this AVP responsive epithelium. All our studies were performed in the background of phosphodiesterase inhibition (MIX) and our results therefore reflect alterations in cAMP formation. A potential effect of calcium on the rate of cAMP degradation was not evaluated in these experiments.

Our initial studies were directed at assessing whether perturbations in the extracellular concentration of calcium can be shown to alter cAMP formation in these intact cells. Our results demonstrate that acute exposures to media calciums of 0.5 or 2 mM do not significantly alter the dose-response curve to AVP (Fig. 3). Even the removal of calcium from the buffer (2.5 μM) without a preincubation period fails to alter cAMP response (data not shown). Furthermore, increasing extracellular calcium to 4.0 mM for 60 min had no significant effect on vasopressin-stimulated cAMP formation (Fig. 4). As this pronounced change in extracellular calcium was associated with no change whatsoever in cytosolic calcium it is clear that cAMP-forming ability of intact inner medullary collecting tubule cells is insensitive to profound alterations in extracellular calcium concentration.

In sharp contrast to the above, our results point to an important role for intracellular calcium as a modulator of cAMP formation. A number of our observations support this contention. The preincubation of cells for 60 min in a calcium-free buffer (2.5 μM) as well as the addition of EGTA consistently enhanced the response to AVP (Fig. 5).

As seen in Table I, this latter maneuver caused a highly significant decrease in the concentration of intracellular calcium. Although chelation of calcium bound to fura-2 that has leaked out of the cell could produce a similar effect, the results of our studies comparing the effects of EGTA with those of manganese argue strongly against such interpretation of the data. By virtue of its 42-fold greater affinity for fura-2 than that of calcium, manganese is a potent tool for the determination of fura-2 leak. As depicted in Fig. 6, manganese caused a small decrease in the apparent Ca–fura-2 concentration, which remained stable over time. EGTA, on the other hand, caused a larger decrease and one that progressed with time, indicating that EGTA caused a true decrease in intracellular calcium. We derived further evidence against a significant leak of fura-2 from the observation that the addition of manganese to a supernatant from cells loaded with fura-2 that have been allowed to sit at room temperature for 60 min results in no change in fluorescence (data not shown). These observations are in accord with those of Burch and Halushka, who described a similar effect of EGTA on cellular calcium in toad bladder epithelial cells (35).

We also attempted to delineate the kinetics of the hormone-receptor interaction in the absence of calcium. The results shown in Fig. 7 demonstrating that the enhanced effect of the virtual absence of calcium is not on the affinity constant (K_m) but on V_{max} could be interpreted as showing that the attachment of AVP to its receptor is not altered (presumably an extracellular event). Rather, a subsequent step in the regulatory protein-catalytic unit chain of events, which is presumably more sensitive to a change in calcium concentration at an intracellular or membrane domain is involved. In this regard it is of interest that our preliminary results with forskolin, an agent that does not require the receptor for enzyme activation, reveal a similar enhancement in EGTA exposed cells. Furthermore, this observation is in accordance with the reported enhancement of arginine vasotocin-stimulated cAMP production in frog skin depleted of calcium by treatment with EGTA (36). It is therefore increasingly apparent that either cytosolic or membrane calcium exerts an important tonic inhibitory effect on the cell's cAMP forming system—the adenylate cyclase complex.

The notion that cellular calcium is the critical modulator of cAMP production is further strengthened by our experiments involving the calcium ionophore A23187. At a concentration that produced no cellular injury, neither morphologically nor biochemically, the ionophore caused a calcium-dependent decrease in cAMP formation. Note that a small decrease was caused by the ionophore even when extracellular calcium was very low, a setting that as noted above tends to enhance cAMP formation. It is likely that in the presence of the ionophore even this very low extracellular concentration allowed calcium to travel down its concentration gradient into the cell, thereby somewhat decreasing cAMP generation. The effect of ionophore was, however, much more marked and statistically significant only at 1 or 4 mM extracellular calcium. Thus, an agent that is well known to enhance cellular calcium entry markedly suppressed cAMP generation. Whether this effect is due to the presence of the calcium cation per se or is a secondary effect of calcium-mediated changes in the cellular environment (e.g., altered cytosolic pH) cannot be determined from these studies. Note, however, that exposure of the cells to ionophore for only 15 min, which would decrease the likelihood of both nonspecific and toxic effects, resulted in a 53.7% decrease in cAMP formation that is very similar to the 57.6% decrease observed after a 1 h exposure to ionophore ($n = 3$). Likewise, it is possible that an effect of the ionophore to stimulate phospholipase could alter membrane fluidity and inhibit cAMP formation (37), but in view of our results this would also be a calcium-dependent process.

In a final group of studies we investigated whether prostaglandins were the mediators of the calcium-induced changes in AVP-stimulated cAMP production. This possibility seemed particularly attractive as calcium can stimulate PG synthesis in a variety of tissues by activation of phospholipase A_2, and the calcium ionophore A23187 is well recognized as a potent stimulus to PG synthesis. Such an effect was evident in our collecting duct cells as well. In turn, PG have inhibited the hydroosmotic response to AVP (21, 22), and more recently it has been shown that 10^{-11} M PGE_2 inhibits AVP-stimulated cAMP formation (38). Our results demonstrate that the effects of calcium were not dependent on the synthesis of PG nor, for that matter, on any cyclooxygenase product. We used meclofenamic acid to inhibit cyclooxygenase as assessed by PGE_2 levels; the drug inhibited PGE_2 synthesis by 95%. We also demonstrated in experiments involving addition of exogenous PGE_2 that meclofenamic acid did not directly affect cAMP formation by a mechanism independent of its PG inhibitory ability. In the presence of me-

clofenamate, both the enhanced effect of the virtual absence of calcium and the inhibitory effect of ionophore were still present. Thus, the alterations in cAMP formation observed in our studies are due to a direct effect of calcium on the adenylate cyclase complex. The locus of this effect remains to be determined. Calcium could impair cAMP generation by altering the activity of either the stimulatory (N_s) or inhibitory (N_i) guanine nucleotide-binding regulatory proteins or by interfering with the catalytic subunit directly. Furthermore, it is possible that calcium-induced phospholipase activity (either direct or mediated via enhanced protein kinase C activity with phosphorylation of lipomodulin and subsequently enhanced phospholipase activity [37]) could decrease membrane fluidity and thereby impair cAMP generation. It is also of interest to note that cAMP formation in the presence of AVP was not itself enhanced in the PG-inhibited state. Thus, at least in inner medullary collecting tubule cells, PG may not be an important antagonist of AVP-stimulated cAMP generation. This is in concert with the recent observations of Sato and Dunn demonstrating a dose-dependent agonist effect of the prostanoid on cAMP and a failure of acetylsalicylic acid to enhance AVP-stimulated cAMP formation by these cells (39).

Finally, a discussion of the physiologic relevance of our data is in order, particularly with respect to the concentrating defect observed in hypercalcemic states. Alterations in buffer calcium concentration in ranges observable in pathologic conditions of severe hypocalcemia (0.5 mM) or in the inner medulla during severe hypercalcemia (4 mM), do not significantly alter the ability of AVP to increase cellular cAMP generation. Studies on water flux in this range of bath calcium have not been performed, with the exception of the study of Goldfarb (11) in which 3.75 mM calcium in the bath enhanced AVP-mediated water flow. Because our studies examined the effect of calcium on the formation of cAMP but not on postcyclic events, the disparity between our study and that of Goldfarb (11) could be due to a stimulatory effect of calcium on postcyclic events (e.g., protein kinase activity, assembly of microtubules or microfilaments). It should be noted that even the lowering of external calcium to 0.027 mM has little effect on AVP-stimulated water movement in the toad bladder (40). Changes in external calcium concentration in the ranges studied herein are not accompanied by alterations in cellular calcium concentration. It is apparent therefore that a perturbation in cellular calcium as those caused by EGTA or ionophore is needed for the cAMP response to be changed also. Water flux studies using EGTA have, to our knowledge, not been reported, while ionophore has been noted to inhibit the response to AVP in most (17, 40) but not all studies (18). In contrast to the observations of Yorio et al. (40), however, we did not find an important role for PG in the effect of the calcium ionophore to inhibit the action of AVP. This may reflect a difference between the toad bladder and inner medullary collecting tubules. Alternatively, PG may act at a site distal to the formation of cAMP (41). If so, a salutory effect of PG inhibition would not be evident in our system.

It is interesting to speculate whether the abnormal urinary concentration that accompanies hypercalcemia is due to an increase in cellular calcium that in turn reduces cAMP formation. Possibly, the prolonged elevation of serum calcium in conjunction with a naturally occurring ionophore could bring about such a change. Alternatively, the primary effect of hypercalcemia could be on the process of generation of interstitial hypertonicity by an effect of the cation on the ascending limb of Henle's loop (35, 42). Our experiments suggest that an additional decrease in the response of the collecting duct to AVP requires the translation of extracellular hypercalcemia to an increase in cellular calcium content.

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