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LABORATORY INVESTIGATION

Extracellular urea concentration modulates cAMP production in the mouse MTAL

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Extracellular urea concentration modulates cAMP production in the mouse MTAL. Ionic reabsorption along the ascending limb of Henle's loop (TAL) is controlled by hormonal stimulation. Most of the hormones that affect this reabsorption regulate ionic transporter activity via cAMP, and some of these hormonal actions have been shown to be modulated by interstitial osmolarity. We studied the early effects of increasing extracellular urea concentration on the production of cAMP induced by arginine vasopressin (AVP) and forskolin in a suspension of medullary portions of TAL (MTAL) prepared from mouse kidney. The addition of urea, performed fifteen minutes before adenylyl cyclase stimulation, decreased both AVP- and forskolin-induced cAMP production. This effect, observed both in the presence and the absence of phosphodiesterase inhibition, was optimal with 300 mmol/liter urea. Addition of urea to the extracellular medium disturbed several cellular parameters, but the decrease in cAMP production appeared to be mediated by the activation of both the protein kinase A and a phosphatase rather than by the modifications in phospholipid metabolism. Since cAMP is the major cytosolic transductional factor in MTAL cells, urea present in the medullary interstitium may thus be considered as an important modulator of hormonal actions in this segment of the nephron.

Renal medullary interstitium, compared to the interstitium of other tissues, is special in several important respects. Its osmolarity is not only extremely high, but it also varies with the hydroosmotic state of the organism and even serves an essential regulatory role in the urinary concentration process. The physiology of medullary cells is adapted to this interstitial environment, one case being the cells of the medullary thick ascending limb of Henle (MTAL), which regulate their ionic reabsorptive properties according to the interstitial osmolarity; NaCl and bicarbonate reabsorption are, for example, reduced by extracellular hyperosmolarity [1–3] via variations in the activity or functional characteristics of some transmembrane transport systems [1, 2]. Some of these modulations are linked to variations in cell volume provoked by extracellular solutes like NaCl [4, 5], but high interstitial urea concentration also affects a large number of molecular reactions without modifying cell volume [6]. The cytoplasmic concentration of organic osmolytes, and particularly of methylamines, which neutralize the denaturing action of high urea concentration on proteins, plays a major role on this respect [6]. The synthesis of these compounds may alter many aspects of metabolism. In particular, the accumulation of glycerophosphocholine (GPC), which derives from phosphatidylcholine via tissue-specific activation of various phospholipases, directly affects the metabolism of phospholipids [6, 7]. Osmosensors have recently been found on various cells [8, 9], and their activation by urea concentrations above 100 mmol/liter triggers activation of MAP (mitogenic activating proteins) kinase-like series of tyrosine/threonine/serine kinases [10]. The various phenomena (accumulation of osmolytes, among which is GPC, inhibition or activation of phosphatases and kinases able to modulate the actions of several cytoplasmic or membrane proteins) involved in the effects of high extracellular urea concentrations may derive from the early activation of these osmosensors [11–14].

On the other hand, in the MTAL, the effects of hormones like arginine-vasopressin (AVP), which control ionic reabsorption, vary with the osmolarity of the extracellular space [15], and the production of cAMP, which mediates these hormonal actions, may be modulated by cytosolic enzyme activities [16]. In a previous study performed on the mouse MTAL [17], we have found that hypertonic NaCl may stimulate AVP-induced cAMP formation via the secretion and action of endogenous adenosine. On the other hand, AVP-induced cAMP production has been shown to be inhibited by extracellular urea in the inner medullary collecting duct [18], but not in the MTAL [19] of the rat. Indeed, the osmolar control of cAMP metabolism appears to be complex, since simultaneous inhibition of adenylyl cyclase activity and enhancement of cAMP accumulation were found to be provoked by the exposure of isolated rat papillary collecting ducts to an hyperosmolar medium added with both NaCl and urea [20].

By measuring AVP-induced cAMP accumulation in a suspension of mouse MTAL, we looked for its modulation by high extracellular urea concentrations and studied the molecular mechanisms involved in the phenomenon.

Methods

The animals used in this study were male CD1 mice (16 to 20 g) from Charles River (Cleon, France), and collagenase (CLS II) was obtained from Worthington (Freehold, NJ, USA). Urea, AVP, forskolin, isobutylmethylxanthin (IBMX), adenosine deaminase, indomethacin, mepacrine, dithio-(2-nitrobenzoic acid) or DTNB, genistein, herbimycin A, staurosporine, N-[2-(3-(4-Bromophenyl-2-propenyl]-amino)-ethyl]5-isoquinoline sulfonamide or H-89,

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N-(6-aminohexyl)-5-chloro-1-naphtalenesulfonamide or W-7, okadaic acid and calyculin A were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The kits for the cAMP radioimmunoassay were obtained from ERIA (Marnes la Coquette, France), and the tritiated arachidonic acid (213 Ci/mmol) was from Amersham (UK).

The experiments were performed in a saline solution of "normal" extracellular composition (140 mmol/liter NaCl, 350 mOsm/liter) containing 20 mmol/liter HEPES and 0.1% bovine serum albumin. The pH of the solution was adjusted to 7.4, and was not decreased by more than 0.1 U pH by the mild bubbling with 95% O_2—5% CO_2 performed throughout the experiment. To prevent any action of secreted endogenous adenosine, adenosine deaminase (2.5 U/ml) was systematically added to the Ringer's solution. When required, organic solvents (DMSO or ethanol) were used at concentrations inferior or equal to 0.1% and were also added at the same concentration in all the experimental and control solutions.

Preparation of the suspension

The suspension of MTAL was prepared as previously described [17], using an adaptation to mouse kidney of the technique based on the enzymatic digestion of the external part of the inner medulla and the purification, by sieving, of the product of the digestion [21]. The preparation, which contained only MTAL fragments under microscopic examination, behaved like microdissected MTALs in terms of cAMP production (stimulation by AVP and glucagon and inhibition by PGE_2).

Stimulation and measurement of cAMP

The suspension was allowed to recover for one hour after the preparation, and then was delivered to the various experimental samples using a pipet with a large aperture. The experiments began with the addition of IBMX (10^{-3} mol/liter) and extra adenosine deaminase (2.5 IU/ml) together with urea and, when required, inhibitors of kinases or phosphatases. Fifteen minutes later, the tubules were stimulated with AVP or forskolin for 10 minutes. The reaction was stopped by addition of ice-cold ethanol (added with 5% formic acid) and cooling. The samples were then evaporated and resuspended in water (with 5-fold dilution), and their cAMP content was determined by radioimmunoassay after an acetylation procedure, according to the manufacturer's instructions. For each measurement, several series of cAMP standards were prepared in the Ringer solution with and without the various urea concentrations tested in the experiments. Each determination was the mean of two results obtained in the same experiment, and all were referred to the protein content of the samples, which was determined according to Lowry [22] on aliquots of the suspension. The protein contents determined throughout the study ranged from 23.2 to 28.5 μg.

Efflux of arachidonic acid

Titrated arachidonic acid (added at the concentration of 1 μCi/ml in both media) was allowed to enter the cells throughout the tissue digestion and the recovery period (2 hr). After washing, the tubules were resuspended as usual, and the urea action tested after a 30 minutes of re-equilibration. The reaction was stopped by addition of an ice-cold Ca^{2+}-free saline solution containing EDTA (10^{-3} mol/liter) and immediate centrifugation (5000 g, 5 seconds), and the released radioactivity was measured in an aliquot of the supernatant.

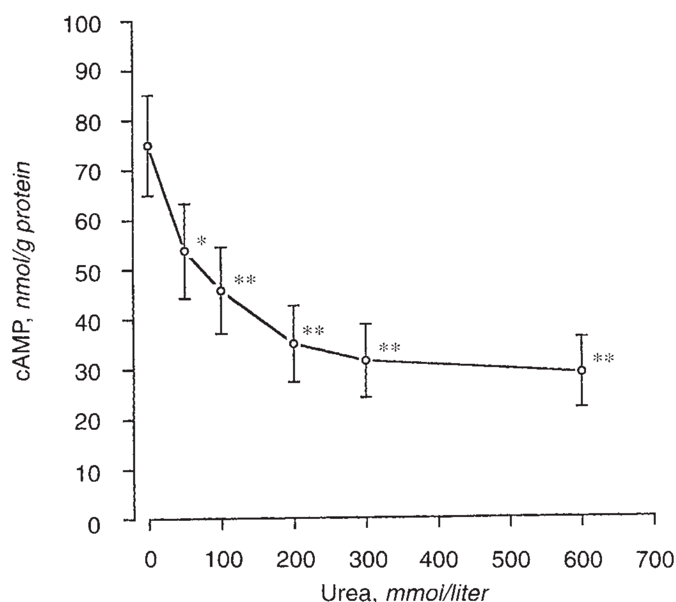

Fig. 1. *Effect of increasing concentrations of urea on the cAMP accumulation induced by a 10 minute incubation with 10^{-9} mol/liter AVP in the presence of IBMX (10^{-3} mol/liter).* Each value, expressed as nmoles cAMP per g tubule protein, is the mean ± SEM of 6 different determinations. Statistical significancy determined by ANOVA completed by Student's t-test versus basal cAMP accumulation; $*P < 0.05$; $**P < 0.01$.

Result analysis

The results are given as mean ± SEM of the number (N) of different determinations. The statistical significance of the differences was established by one-way analysis of variance (ANOVA) and Student's t-test (unpaired, excepted for measurements of arachidonic acid effluxes).

Results

Action of extracellular urea on adenylyl cyclase in the suspension of mouse mTAL

Under our experimental conditions, no significant effect of urea could be observed without AVP stimulation, since in that case the amount of cAMP in the suspension was at the limit of the RIA detection. On the other hand, the addition of urea to the incubation medium decreased the AVP-induced cAMP accumulation. The maximal effect of urea was reached at a concentration of 300 mmol/liter and corresponded to a 50% inhibition: the 10-minute cAMP accumulation induced by 10^{-9} mol/liter of AVP was 74.8 ± 11.6 nmoles cAMP per gram of tissue protein in tubules suspended in control Ringer solution, but only 32.5 ± 8.1 nmoles cAMP per gram of tissue protein in the presence of 300 mmol/liter urea ($N = 6$; Fig. 1). The urea concentration leading to half-maximal inhibition was approximately 50 mmol/liter.

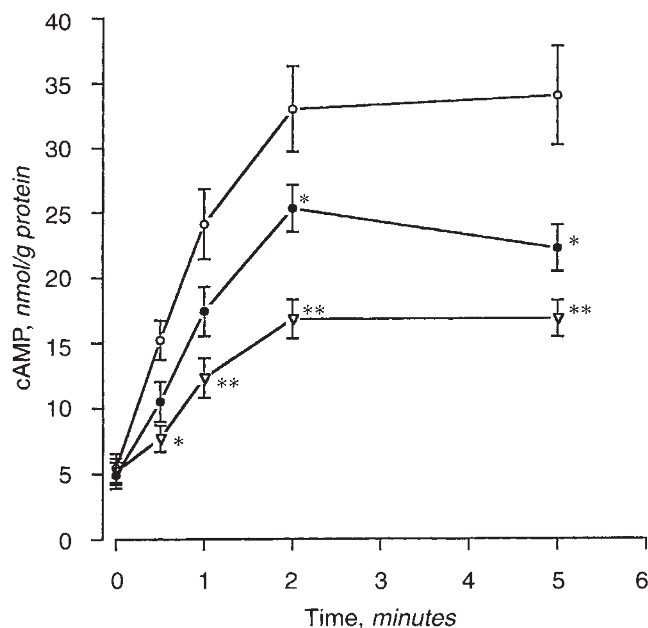

Fig. 2. *Production of cAMP induced by 10^{-8} mol/liter of AVP in the absence of IBMX in control medium (○) and after the addition of 100 (●) or 300 (▽) mmol/liter of urea.* Urea was added together with adenosine deaminase, 15 minutes before time 0, which corresponded to the addition of AVP. Each value is the mean ± SEM of 5 different determinations. Effect of urea, $^*P < 0.05$, $^{**}P < 0.01$ versus control (unpaired Student's *t*-test).

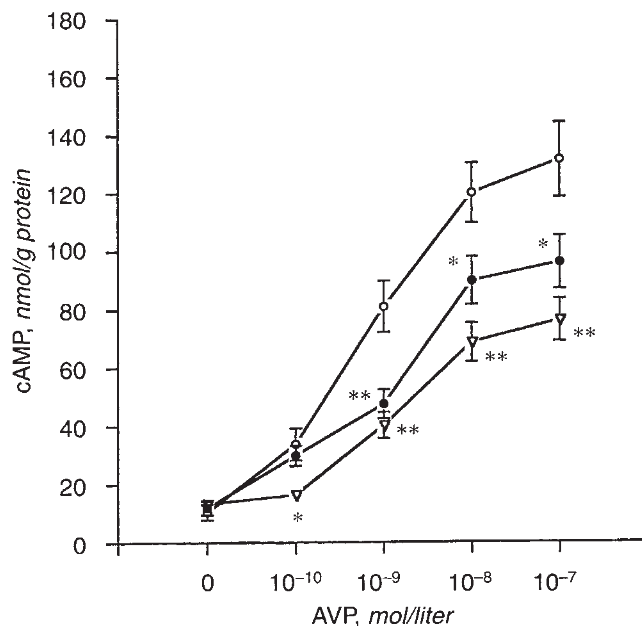

Fig. 3. *Cyclic AMP accumulated in the presence of IBMX (10^{-3} mol/liter) during 10 minutes of stimulation with increasing concentrations of AVP in the control medium (○) and after addition of 150 mmol/liter NaCl (●) or 300 mmol/liter urea (▽).* Each value is the mean ± SEM of 6 different determinations. Effect of the hyperosmotic solutions, $^*P < 0.05$, $^{**}P < 0.01$ versus control (unpaired Student's *t*-test).

Most experiments were performed in the presence of 10^{-3} mol/liter of IBMX, but the observed inhibition was not due to this addition, since urea also decreased the cAMP production induced by 10^{-8} mol/liter of AVP in the absence of any phosphodiesterase inhibitor (Fig. 2). This shows that the interstitial urea concentration itself may physiologically control cAMP production *in vivo.*

The amount of accumulated cAMP was reduced by hypertonic urea, but the kinetics of the dose-response curve to AVP were not affected (Fig. 3). Both in the absence and the presence of 300 mmol/liter of urea, 10^{-7} mol/liter of AVP induced the maximal accumulation of cAMP, which corresponded to 137.5 ± 11.3 and 70.9 ± 9.8 (N = 6) nmoles cAMP per gram of tubular protein for a 10 minute incubation in control and urea-enriched buffer, respectively. The ED_{50} of the reaction did not appear to be modified by the urea addition and was graphically estimated at 10^{-9} mol/liter AVP at both external osmolarities. Addition of NaCl (150 mmol/liter) to the saline solution also decreased AVP-induced cAMP production. However, since the maximal cAMP accumulation, which was also triggered by 10^{-7} mol/liter of AVP, reached 96.1 ± 9.2 nmoles per gram of tubular protein (N = 6) in the presence of added NaCl, the saline inhibition of the cAMP production was less important than the urea effect.

Addition of urea also decreased the cAMP accumulation induced by 10^{-5} mol/liter of forskolin (Fig. 4). As was observed with AVP, the addition of 300 and 50 mmol/liter urea led to maximal and half-maximal inhibition, respectively, of the cAMP production triggered by forskolin. The maximal effect of urea was also a 50% inhibition, since the 10 minute accumulation of cAMP induced by forskolin was 178 ± 19 and 322 ± 21 nmoles cAMP per gram of tubular protein (N = 6) with and without 300 mmol/liter added urea, respectively.

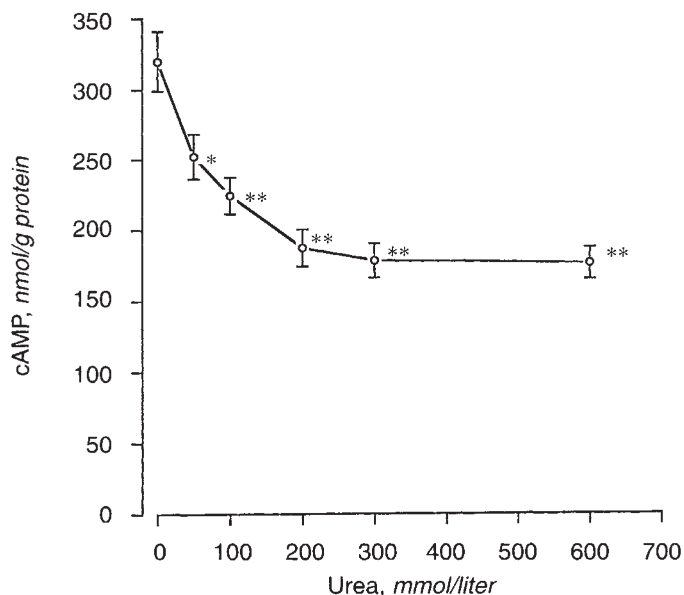

Fig. 4. *Effect of urea on the cAMP accumulation induced by a 10 minute incubation with 10^{-5} mol/liter of forskolin in the presence of IBMX (10^{-3} mol/liter).* Each value is the mean ± SEM of 6 different determinations. Statistical significance was determined by ANOVA completed by Student's *t*-test versus basal cAMP accumulation; $^*P < 0.05$; $^{**}P < 0.01$.

Molecular mechanisms involved in the extracellular urea-induced inhibition of cAMP production

Modification of phospholipid metabolism. Glycerophosphocholine, known to accumulate in the cytoplasm of MTAL cells bathed

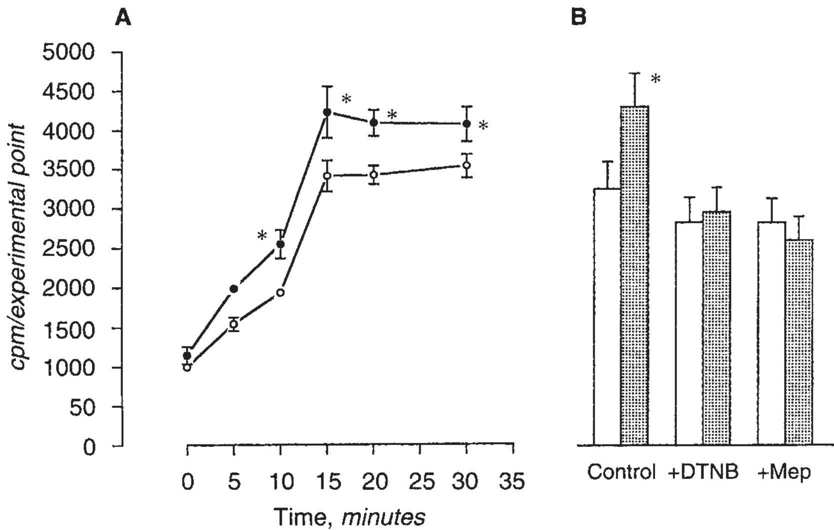

Fig. 5. A. *Time dependence of the release of radioactivity in the control medium (○) and after addition of 300 mmol/liter urea (●) at time 0.* **B.** Release of radioactivity measured after a 20 minute incubation in the control medium (white columns) and in the presence of 300 mmol/liter urea (grey columns) without or with either 10^{-4} mol/liter of DTNB or 5.10^{-5} mol/liter of mepacrine (Mep). Each value is the mean ± SEM of 5 different determinations. Effect of urea addition, $*P < 0.05$ versus control (paired Student's *t*-test).

by an hypertonic medium, may derive from phosphatidylcholine hydrolysis, and its production may alter arachidonic acid metabolism. In order to show such an early urea-induced modification, titrated arachidonic acid was preincorporated into the tissues throughout the digestion and recovery periods. Resuspension of the tubules in an arachidonic acid-free medium then triggered a rapid release of radioactivity, which after fifteen minutes corresponded to 12.8 ± 2.5% ($N = 6$) of the initial tissue content. The addition of 300 mmol/liter urea to the medium significantly increased the radioactive efflux by 30%, and the effect of urea was abolished in the presence of 10^{-4} mol/liter of either mepacrine or DTNB, which both inhibit phospholipase A_2 activity (Fig. 5) [23]. Hypertonic urea thus appears to stimulate an early phospholipid hydrolysis, but the nature of the released tritiated compound was not determined; since the labeling period was too short to allow isotopique equilibrium, the phenomenon could not be well defined.

The following experiments were performed to determine the involvement of released arachidonic acid or arachidonate metabolites in the urea-induced inhibition of cAMP production. Indeed, exogenous arachidonic acid (10^{-6} and 5.10^{-6} mol/liter) decreased the amount of cAMP produced by AVP in the mouse MTAL suspension (Fig. 6). However, the inhibitory actions of the optimal extracellular urea concentration (+300 mmol/liter) and either concentration of exogenous arachidonic acid were additive. The effect of urea thus did not appear to be mediated by released endogenous arachidonic acid. The cAMP accumulation induced by AVP (10^{-9} mol/liter) and forskolin (10^{-5} mol/liter) without and with urea was also measured in the presence of inhibitors of cyclooxygenase (indomethacin 10^{-6} mol/liter), lipoxygenase (esculetin 10^{-4} mol/liter) and P-450 monooxygenases (metyrapone 10^{-4} mol/liter). Neither inhibitor antagonized the action exerted by urea on adenylyl cyclase, and phospholipase A_2 inhibition by mepacrine and DTNB was also ineffective (Table 1). The urea-induced modifications in lipid metabolism shown by the radioactive efflux therefore did not appear to be the cause of the decreased cAMP production.

Role of phosphorylations: Inhibition of kinases. The cyclic AMP accumulated under basal conditions and under stimulation with

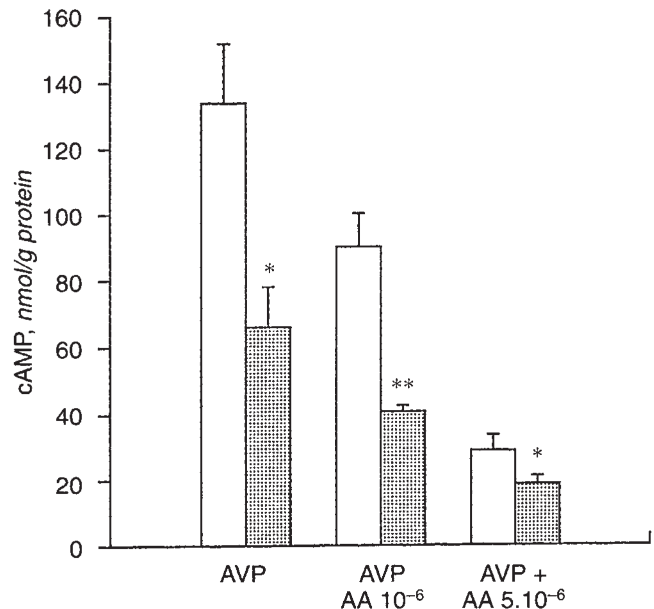

Fig. 6. *Accumulation of cAMP induced by 10 minutes of incubation with 10^{-9} mol/liter of AVP under control conditions (white columns) and in the presence of 300 mmol/liter of urea (grey columns) in the absence and the presence of exogenous arachidonic acid.* Each value is the mean ± SEM of 5 different determinations. Effect of urea, $*P < 0.05$, $**P < 0.01$ versus control (unpaired Student's *t*-test).

AVP (10^{-9} mol/liter) and forskolin (10^{-5} mol/liter) in saline solution with or without 300 mmol/liter of added urea was then measured in the presence of H-89 (10^{-6} mol/liter), W-7 (10^{-6} mol/liter), herbimycin A (5.10^{-6} mol/liter) and genistein (5.10^{-5} mol/liter), which respectively inhibited the cAMP-dependant kinase (PKA), the Ca^{2+}/calmodulin dependant kinase and, for the latter two, tyrosine kinases [24]. Staurosporine, a known inhibitor of protein kinase C, was tested at the concentrations of 10^{-8} and 10^{-6} mol/liter, since it antagonized both PKA and PKC at high concentrations. Neither under control conditions nor in the

Table 1. cAMP accumulation (nmol/g protein)

[Urea] *mmol/liter*	AVP *10^{-9} mol/liter* 0	AVP *10^{-9} mol/liter* 300	FSK *10^{-5} mol/liter* 0	FSK *10^{-5} mol/liter* 300
Control	78.5 ± 9.6	41.7 ± 6.4	318 ± 24	193 ± 19
Mepacrine *5 × 10^{-5} mol/liter*	73.3 ± 11.1	36.8 ± 6.2	315 ± 21	189 ± 18
DTNB *10^{-4} mol/liter*	70.1 ± 9.7	35.3 ± 6.2	298 ± 19	163 ± 17
Indomethacin *10^{-6} mol/liter*	75.2 ± 10.5	37.3 ± 8.0	305 ± 18	177 ± 20
Esculetin *10^{-4} mol/liter*	78.3 ± 10.1	39.5 ± 6.4	313 ± 19	184 ± 18
Metyrapone *10^{-4} mol/liter*	74.6 ± 9.5	38.7 ± 5.7	299 ± 21	181 ± 23

Effect of mepacrine (5 [times] 10^{-5} mol/liter), DTNB (10^{-4} mol/liter), indomethacine (10^{-6} mol/liter), esculetin (10^{-4} mol/liter) and metyrapone (10^{-4} mol/liter) on the cAMP accumulation induced by a 10 minute incubation with AVP (10^{-9} mol/liter) and forskolin (10^{-5} mol/liter) in control and urea-enriched medium. Each value represents the mean ± SEM of 4 different determinations. The data obtained with each inhibitor was statistically tested against the control cAMP production by the unpaired Student's *t*-test: $P > 0.05$.

Table 2. cAMP accumulation (nmol/g protein)

[Urea] *mmol/liter*	AVP *10^{-9} mol/liter* 0	AVP *10^{-9} mol/liter* 300	FSK *10^{-5} mol/liter* 0	FSK *10^{-5} mol/liter* 300
Control	78.6 ± 8.0	30.6 ± 3.5	320 ± 29	183 ± 18
Genistein *5 × 10^{-5} mol/liter*	75.5 ± 8.2	32.7 ± 3.5	303 ± 31	166 ± 16
Herbimycin A *5 × 10^{-6} mol/liter*	77.3 ± 9.1	30.5 ± 4.1	326 ± 32	192 ± 20
Staurosporine *10^{-8} mol/liter*	79.6 ± 8.3	34.1 ± 3.8	315 ± 35	177 ± 23
W-7 *10^{-6} mol/liter*	69.7 ± 8.5	28.8 ± 3.4	293 ± 30	166 ± 18

Effect of genistein (5 [times] 10^{-5} mol/liter), herbimycin A (5 [times] 10^{-6} mol/liter), staurosporine (10^{-8} mol/liter) and W-7 (10^{-6} mol/liter) on the cAMP accumulation induced by a 10 minute incubation with AVP (10^{-9} mol/liter) and forskolin (10^{-5} mol/liter) in control and urea-enriched medium. Each value represents the mean ± SEM of 4 different determinations. The data obtained in the presence of each kinase inhibitior was statistically tested by the unpaired Student's *t*-test versus the control cAMP production: $P > 0.05$.

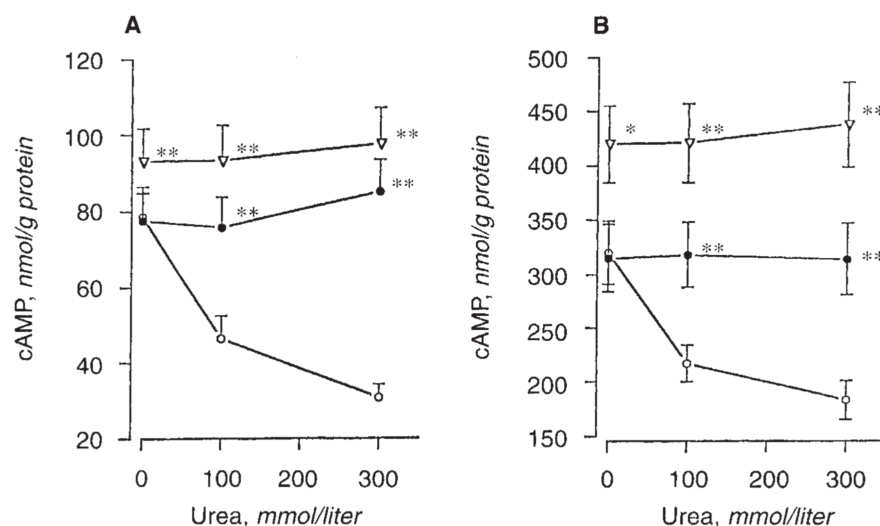

Fig. 7. *Accumulation of cAMP induced by a 10 minute stimulation with 10^{-9} mol/liter of AVP* (**A**) *and 10^{-5} mol/liter of forskolin* (**B**), *under control conditions* (○) *and in the presence of 10^{-6} mol/liter of H-89* (●) *and staurosporine* (▽). Each value is the mean ± SEM of 5 different determinations. Effect of the inhibitors, $^{*}P < 0.05$, $^{**}P < 0.01$ versus control (unpaired Student's *t*-test).

presence of 300 mmol/liter urea did genistein and herbimycin A, 10^{-8} mol/liter of staurosporine and W-7 modify AVP- or forskolin-induced cAMP production (Table 2). On the other hand, H-89 suppressed the urea-induced inhibition of cAMP production observed with both AVP and forskolin (Fig. 7), which suggests the involvement of PKA in the phenomenon. Staurosporine at the concentration of 10^{-6} mol/liter reproduced the effect of H-89, but also stimulated the production of cAMP induced by AVP (+18%) and forskolin (+30%), both in control and urea-enriched solutions. Like that of H-89, the staurosporine-induced suppression of the inhibitory urea effect may derive from PKA inhibition, but this finding also suggests that PKC decreases adenylyl cyclase activity both under basal conditions and in the presence of hypertonic urea.

Inhibition of phosphatases. We also tested the effects of two protein phosphatase inhibitors: okadaic acid and calyculin A. Both inhibit phosphatase 2A with an ID_{50} of about 10^{-8} mol/liter, whereas only the latter significantly depresses phosphatase 1 at the concentration of 2.10^{-9} mol/liter [24]. The inhibitory effect exerted by urea on cAMP formation induced by AVP and forskolin was suppressed by 10^{-8} mol/liter of both compounds (Fig. 8), but was unaffected by both compounds tested at the

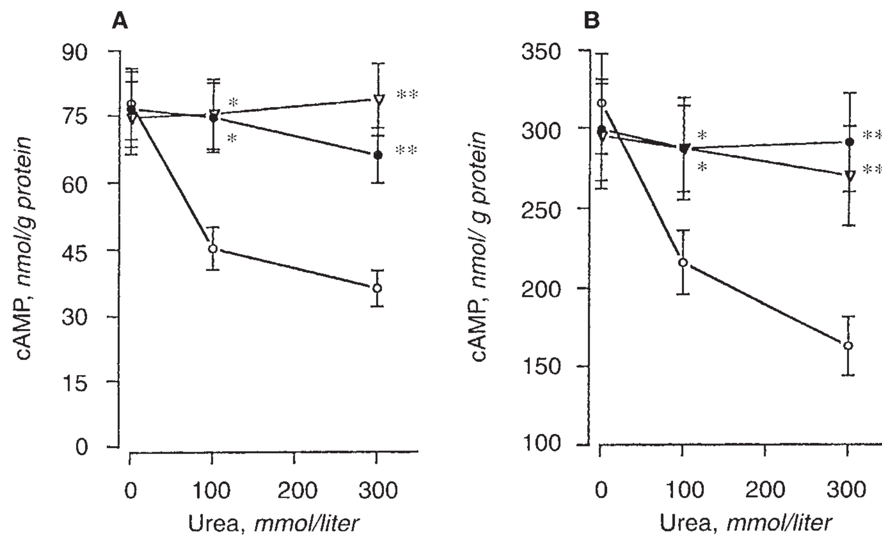

Fig. 8. *Accumulation of cAMP induced by 10 minutes of stimulation with 10^{-9} mol/liter of AVP* (**A**) *and 10^{-5} mol/liter of forskolin* (**B**) *under control conditions* (○) *and in the presence of 10^{-8} mol/liter of okadaic acid* (●) *and calyculin A* (▽). Each value is the mean ± SEM of 5 different determinations. Effect of the inhibitors, $^{*}P < 0.05$, $^{**}P < 0.01$ versus control (unpaired Student's *t*-test).

concentration of 2.10^{-9} mol/liter. The urea effect thus appears to be mediated by activation of a protein phosphatase.

Discussion

Although our experimental conditions did not permit the study of modulations of unstimulated cAMP production (which is at the limit of detection of the RIA), our results do show that hypertonic urea inhibits AVP-induced cAMP accumulation in mouse mTAL. The phenomenon may occur physiologically *in vivo*, since it is respectively half-maximal and maximal with 50 and 300 mmol/liter urea (which are concentrations encountered in the renal interstitium), and is also observed in the absence of phosphodiesterase inhibition. However, the regulation of cAMP accumulation by extracellular hyperosmolarity appears to be a complex process. This was first shown by an analysis of the effect of increasing extracellular osmolarity by the addition of NaCl and urea on both adenylyl cyclase activity and cAMP accumulation in the papillary collecting duct of the rat [20]. The authors found both decreased adenylyl cyclase activity and enhanced cAMP accumulation in the presence of osmolarities varying between 200 and 2000 mOsm/liter. In contrast, AVP-stimulated cAMP accumulation was recently shown to be inhibited by the addition of 200 mol of urea per kg of water in isolated rat inner medullary collecting duct [18], and, in the rat MTAL, no significant decrease in AVP-induced cAMP production was found upon the addition of 400 mmol/liter of extracellular urea [19]. This apparent contradiction between the latter results and ours may be due to inter-species differences, but may also be the result of differences in experimental protocols, and particularly because we added adenosine deaminase and IBMX together with urea throughout our study. Indeed, when used as an inhibitor of the phosphodiesterases, IBMX also inhibits the P_1 purinergic receptors, and we have previously observed an adenosine-mediated autocrine up-regulation of adenylyl cyclase activity in the mouse mTAL suspension exposed to hypertonic NaCl [17]. The functional importance of endogenous adenosine and its possible late actions was confirmed by the fact that addition of NaCl did not produce a significant variation in AVP-induced cAMP accumulation measured in the presence of IBMX when the tubule suspension was prepared without added adenosine deaminase [17]. The effects of the addition of IBMX on the variations in cAMP production brought about by hyperosmolarity already noticed by others [25] may therefore also depend on the timing of the xanthine addition, which might explain discrepancies in the literature. Nonetheless, the existence of other endogenous factors able to control the complex reactions involved in the phenomenon cannot be rejected.

The decrease in cAMP production induced by extracellular hypertonicity in the present study is independent of adenosine. Its induction by urea, which does not modify the cell volume, demonstrates that it is also not linked to volume variations. On the other hand, this "intrinsic" action of hypertonicity may take part in the inhibition triggered by NaCl supplementation, which presents the same charactristics as the urea inhibition, and be partly inhibited by the cell volume variations since cAMP production is decreased more by urea than by the addition of NaCl.

Hypertonicity has been shown to down-regulate hormonal receptors. The phenomenon has been decribed for AVP receptors on A6 cells exposed to a sucrose-enriched medium [25] and for endothelin A receptors in rat medullary interstitial cells [26]. Such a phenomenon is not involved in the urea-induced decrease in cAMP production observed in the MTAL suspension, since the inhibition is similar for both AVP and forskolin agonists. As shown by the absence of any shift of the dose-response curve of AVP-induced cAMP accumulation, urea also does not act by depressing V_2 receptor sensitivity. On the other hand, the inhibition by urea of the forskolin stimulation clearly shows that a functional modulation of a G protein is not involved in the reduction of the cAMP production. The catalytic subunit of adenylyl cyclase thus appears to be the site of the inhibitory action of hypertonic urea.

The molecular reactions triggered by high extracellular concentrations of urea appear to be complex. The stimulation of the radioactive efflux induced by the addition of 300 mmol/liter urea in the mTAL suspension strongly shows that some modification in phospholipid metabolism rapidly occurs in response to the exposure to hypertonic urea. Unfortunately, the radioactive labeling that can be performed on the tubule suspension is too short to

yield isotopic equilibrium and too weak to allow the determination of the origin of the released tritiated compound. Therefore, no conclusion can be reached concerning an early hydrolysis of arachidonyl-phosphatidylcholine. Such a compound was shown to not produce glycerophosphocholine (GPC) directly in MDCK cells that were subjected to hypertonic urea [12]. Furthermore, GPC accumulation is a very slow process that requires at least several hours or days according to the experimental model [6, 12, 27]. The release of arachidonic acid or metabolites observed in the very first minutes following addition of external urea to the MTAL suspension thus cannot be directly related to GPC synthesis, and its physiological function remains unclear. It does appear to derive from an activation of some phospholipase A_2, since it is suppressed by the addition of mepacrine and DTNB, and such a reaction is indeed included in the MAP kinase-like reactive pathway that has been implicated in the cell response to hyperosmolarity [11, 28, 29].

The released radioactivity may be born either by arachidonic acid or by some of its metabolites. Like in rat MTAL [30], external arachidonic acid inhibits AVP-induced cAMP formation in mouse mTAL. However, the effects of exogenous arachidonic acid and urea are additive, and the urea-induced decrease in cAMP accumulation thus cannot be related to the release of endogenous arachidonic acid. Metabolites of the latter compound also do not seem to be involved in the phenomenon. Indeed, the major degradation pathway of arachidonic acid in MTAL is that triggered by the activation of P-450 monooxygenases [31], and it does not appear to be involved in the adenylyl cyclase inhibition, since the addition of metyrapone had no effect. The negative results obtained in the presence of indomethacin and esculetin also rule out any induction by extracellular urea of cyclooygenase or lipoxygenase that could produce compounds inhibiting cAMP production. Modulation of phospholipid metabolism triggered by the addition of urea in the mouse MTAL suspension therefore does not appear to be implicated in the decrease in cAMP production.

On the other hand, alterations in the phosphorylation/dephosphorylation processes appear to support the hypertonic urea-induced inhibition of adenylyl cyclase. The negative results obtained with W-7 on the one hand, and genistein and herbimycin A on the other, argue against the involvement of either a Ca^{2+}/calmodulin sensitive- or a tyrosine kinase. By contrast, the suppression of the urea-induced inhibition of cAMP production provoked by the addition of H-89 demonstrates that this inhibition is mediated by activation of the cAMP-dependent protein kinase, and this conclusion is reinforced by the effect of 10^{-6} mol/liter staurosporine. Other PKA-mediated effects of extracellular urea have been found in other experimental models [32], and a negative feedback of the adenylyl cyclase catalytic subunit by PKA has also been shown [16]. A protein kinase C-dependent negative control of types I, II and III adenylyl cyclase activity has also been described [16], and might support the stimulation of cAMP production induced by 10^{-6} mol/liter of staurosporine that was found in the present study, in both iso- and hyperosmotic media. However, this regulation would not be implicated in the action of urea and its existence was not confirmed by the negative result obtained with 10^{-8} mol/liter of staurosporine.

The urea-induced intracellular modifications that control adenylyl cyclase activity are still more complex, since they are also antagonized by the inhibition of phosphatases. This is shown by the results obtained in the presence of okadaic acid and calyculin A. The suppression of the inhibition of cAMP production induced by 100 and 300 mmol/liter of urea observed in the presence of 10^{-8} mol/liter of either antagonist suggests the involvement of a phosphatase 2A in the hypertonic urea action. Complex regulatory mechanisms based on sequential actions of phosphorylations and dephosphorylations have been described in several models, for example in the control of the $Na^+/K^+/2Cl^-$ cotransporter [33] and the Na^+/H^+ antiport [34]. Such a reactive pathway appears to be involved in physiological regulation of adenylyl cyclase activity by extracellular urea concentration in the mouse MTAL.

Further analysis of the enzyme activities, which are difficult to perform on renal tubules, would be required to better define the cascade of reactions triggered by external hypertonicity in MTAL. The present study shows that physiological interstitial urea concentration may inhibit adenylyl cyclase in this segment of nephron, and that this action derives rather from alterations in protein phosphorylation than from modifications of phospholipid metabolism. Since cAMP is the major mediator of hormonal action in MTAL, medullary interstitial urea concentration may thus be considered an important modulator of the tubular ionic reabsorption.

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