Inhibitory Guanosine Triphosphate-binding Protein-mediated Regulation of Vasopressin Action in Isolated Single Medullary Tubules of Mouse Kidney

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Abstract

Vasopressin (AVP) plays a key role in maximal urine concentration by stimulating NaCl reabsorption in the medullary thick ascending limbs of Henle (MAL) and by increasing water permeability in the medullary collecting tubules (MCT). These effects of AVP in MAL and MCT are mediated by cAMP. Alpha$_2$-adrenergic stimulation in MCT, and high ambient Ca^{2+} and PGE_2 in MAL inhibit AVP-dependent cAMP production and thereby modulate urine concentration. The present study was undertaken to clarify the mechanisms underlying the inhibition of AVP-dependent cAMP production by these agents using microdissected mouse MAL and MCT. Preincubation of MCT and MAL with 1 μg/ml pertussis toxin for 3 and 6 h, respectively, resulted in ADP-ribosylation of an ~ 41-kD protein, which was presumably an alpha subunit of the inhibitory GTP-binding protein G_i. Epinephrine, 10^{-6} M, via alpha$_2$-adrenergic stimulation, inhibited AVP-dependent cAMP production in MCT. Preincubation of MCT for 3 h with pertussis toxin abolished the inhibition of AVP-dependent cAMP production by epinephrine. High ambient Ca^{2+} and PGE_2 both inhibited AVP-dependent cAMP production in MAL. Preincubation of MAL for 6 h with pertussis toxin abolished the inhibition by high ambient Ca^{2+} and attenuated the inhibition by PGE_2. Preincubation of MCT or MAL with pertussis toxin for 1 h was ineffective in ADP-ribosylation and did not modify the inhibition of AVP-dependent cAMP production by these agents in both nephron segments. Our data suggest that the inhibition of AVP-dependent cAMP production by alpha$_2$-adrenergic stimulation in MCT, and by high ambient Ca^{2+} and PGE_2 in MAL, is mediated, at least in part, through activation of G_i.

Introduction

Vasopressin (AVP)[1] plays a central role in maximal urine concentration by stimulating NaCl reabsorption in medullary thick ascending limbs of Henle (MAL), thus generating the medullary hypertonicity by increasing the water permeability of collecting tubules and allowing water absorption from the urine along the osmotic gradient. These effects of AVP in both nephron segments are mediated by activation of adenylate cyclase (1–8). Activation of adenylate cyclase by a hormone involves binding of the hormone to its specific receptor, activation of stimulatory GTP-binding protein G_s, and activation of the catalytic unit of the enzyme. Several hormones or factors are known to inhibit adenylate cyclase activity through activation of inhibitory GTP-binding protein G_i (9).

Several agents have been reported to modulate urine concentration by inhibiting AVP-dependent cAMP production in collecting tubules and MAL. Thus, it has been shown that alpha$_2$-adrenergic agonists inhibit AVP-dependent cAMP production and thereby suppress water permeability in collecting tubules, while PGE_2 and high ambient Ca^{2+} inhibit AVP-dependent cAMP production in MAL (10–14). Indeed, PGE_2 suppresses AVP-stimulated NaCl reabsorption in MAL by inhibiting AVP-dependent cAMP production (15).

The mechanisms underlying the inhibition of AVP-dependent cAMP production by these agents have been poorly understood. Nonetheless, according to the data obtained in other tissues (16, 17), it is likely that the effects of alpha$_2$-adrenergic stimulation in collecting tubules are mediated through the activation of inhibitory GTP-binding protein G_i of the adenylate cyclase system. It is possible that the inhibition of AVP-dependent cAMP production by high ambient Ca^{2+} and PGE_2 might also be mediated through G_i. Thus, our present study was undertaken to examine whether these agents inhibit AVP-dependent cAMP production in renal tubules through the activation of G_i. To this end, we used pertussis toxin, an agent known to specifically inhibit G_i-mediated suppression of adenylate cyclase activity by ADP-ribosylation of the alpha subunit of G_i (18). The results of our study suggest that alpha$_2$-adrenergic stimulation in MCT, and PGE_2 and high ambient Ca^{2+} in MAL suppress AVP-dependent cAMP production through the activation of G_i.

Methods

Synthetic AVP (grade VI), collagenase (type 1), (−)-epinephrine, DL-propranolol, and yohimbine were purchased from Sigma Chemical Co. (St. Louis, MO). ^{32}P-NAD was purchased from New England Nuclear (Boston, MA). Pertussis toxin (islet-activating protein) was purchased from Kaken Seiyaku (Tokyo).

Isolation of single nephron segments was performed in a fashion similar to that reported previously from our laboratory with minor modifications in the buffer media used (13, 14, 19, 20). In the present study, we used Dulbecco's modified Eagle-minimal essential medium (DMEM) containing 0.1% BSA, 24 mM $NaHCO_3$, and 25 mM Hepes, pH 7.4, equilibrated with 95% O_2 and 5% CO_2 (DMEM-Hepes medium). Male ICR mice weighing ~ 30 g were lightly anesthetized with an intraperitoneal injection of sodium pentobarbital, 2 mg/mouse. The left kidney was perfused with ice-cold DMEM-Hepes medium containing 0.1% collagenase. The left kidney was removed and slices of

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1. *Abbreviations used in this paper:* AVP, vasopressin; MAL, medullary thick ascending limbs of Henle; MCT, medullary collecting tubules; MIX, methylisobutylxanthine; G_i, inhibitory GTP-binding protein; G_s, stimulatory GTP-binding protein.

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~ 0.5–1.0-mm thickness were incubated at 37°C for 10 min in the same medium under 95% O_2 and 5% CO_2. After collagenase treatment, kidney slices were rinsed extensively with ice-cold dissection medium (see below). MAL and MCT were dissected from the outer medulla under a stereomicroscope in ice-cold (0–5°C) dissection medium. The composition of the dissection medium was (in millimolars): NaCl, 137; KCl, 5; Na_2HPO_4, 0.33; KH_2PO_4, 0.44; $MgSO_4$, 0.8; $MgCl_2$, 1.0; $CaCl_2$, 0.25; glucose, 5.5; and Hepes, 10; pH 7.4, and with 0.025% BSA.

In vitro ADP-ribosylation was examined in accordance with the methods described by Okajima et al. (21) with slight modifications. Appropriate pieces of MAL and MCT were transferred with 10 μl of dissection medium into 100 μl DMEM-Hepes buffer with or without 1 μg/ml pertussis toxin placed on the bottom of siliconized biological slides kept on ice. Tubular length was measured through a drawing tube attached to the microscope as reported previously (13, 14, 19, 20). After preincubation under 95% O_2 and 5% CO_2 at 37°C with a moist atmosphere for varied lengths of time as specified in the Results, incubation medium was aspirated and tubules were washed three times with 100 μl DMEM-Hepes medium without pertussis toxin under stereomicroscopic observation. After aspiration of the final rinse, the medium was changed to 20 μl hypotonic solution. The composition of the hypotonic solution was (in millimolars): $MgCl_2$, 5; EGTA, 1; Tris-HCl, 25; pH 7.5, with 1 mg/ml aprotinin. After freezing and thawing repeated three times to permeabilize the tubular cell membrane, the hypotonic medium was changed to 40 μl of reaction medium. The composition of the reaction medium was (in millimolars): Tris-HCl, 25; EGTA, 1; $MgCl_2$, 2.5; ATP, 1; DTT, 2; thymidine, 10; ^{32}P-NAD, 0.01; pH 7.6, with 0.1 mg/ml aprotinin and 25 μg/ml pertussis toxin. Tubules were incubated in the reaction medium at 37°C for 20 min, then cooled on ice, and the medium was changed to 50 μl of 50 mM Tris-HCl buffer, pH 6.8, containing 1% SDS, 2% β-mercaptoethanol, and 10% glycerol. Samples were transferred to test tubes, incubated at 100°C for 5 min, and applied to SDS-PAGE. After electrophoresis, an autoradiogram of the gel was obtained after a 10-d exposure time. At each step of a medium change, the medium was aspirated carefully under stereomicroscope so as not to aspirate the tubules; tubules were then washed two to three times with appropriate medium used for the following procedure.

Incubation of tubules with AVP and the assay of cAMP were performed with slight modifications to those reported previously from our laboratory (13, 14, 19, 20, 22). Immediately after the transfer of appropriate pieces of MAL and MCT with 2-μl dissection medium into 20 μl DMEM-Hepes medium with or without 1 μg/ml pertussis toxin placed on the bottom of a siliconized test tube, the tube was kept in the ice-cold water bath. After measurement of tubular length, the tubules were preincubated at 37°C for several hours under 95% O_2 and 5% CO_2 with a moist atmosphere as specified in the Results. In preliminary studies, we found that evaporation of the medium was < 2% during this incubation period in our experimental condition. After the preincubation with or without pertussis toxin, 20 μl DMEM-Hepes medium containing 2.4 mM methylisobutylxanthine (MIX) (prewarmed to 37°C), AVP, and test agents were added, and the incubation was continued for 3 min. In the experiments of high medium Ca^{2+} concentration, Ca^{2+} concentration was raised only after preincubation, i.e., at the same time of the addition of MIX and AVP. Separate aliquots of tubules were incubated in parallel without AVP but with MIX and test agents to determine the basal cAMP production, i.e., the sum of cAMP produced during preincubation and AVP-independent cAMP production during a 3-min incubation with MIX and test agents. Basal cAMP production without AVP in every experimental condition of the present study was always low (< 10 fmol/mm in MCT and < 5 fmol/mm in MAL), compared with AVP-dependent cAMP production as shown in the Results. After a 3-min incubation with or without AVP, the reaction was terminated by the addition of 50 μl of 10% TCA. The TCA was extracted three times with 1 ml ether saturated with water and the residual aqueous phase was dried at 37–42°C. Samples were kept at −20°C until cAMP determination using a RIA assay kit from Yamasa Shoyu (Tokyo). The alpha-adrenergic stimulation was tested by adding epinephrine together with propranolol. The concentration of AVP used was 10^{-8} M, which is near the maximal concentration needed to stimulate cAMP production in MAL and MCT, based on the results of our previous studies (13, 14, 19, 20). In each experiment, the tubules from the same animal were preincubated for the same length of time with or without pertussis toxin as time-controlled experiments.

The determination of the adenylate cyclase activity was performed in a similar fashion to those previously reported from our laboratory with slight modifications (19). The dissected tubules were permeabilized by freezing and thawing two times in a hypotonic solution. The composition of the hypotonic solution was (in millimolars): Tris-HCl, 8; $MgCl_2$, 1.0; EGTA, 0.25; pH 7.4, with 0.1% BSA. The tubules were transferred with 2 μl of hypotonic solution into 8 μl of prereaction medium placed on the bottom of the siliconized test tube in the ice-cold water bath. The composition of the prereaction medium was (in millimolars): Tris-HCl, 100; $MgCl_2$, 3.8; EGTA, 0.25; MIX, 1.5; pH 7.4, with 0.1% BSA, and different Ca^{2+} concentrations. Final concentrations of Ca^{2+} added are shown in the Results. After measurement of the tubular length, 10 μl of reaction medium was added and tubules were incubated for 30 min at 30°C. The composition of the reaction medium was (in millimolars): Tris-HCl, 100; $MgCl_2$, 3.8; EGTA, 0.25; MIX, 1.5; ATP, 1.2; phosphocreatine, 40; creatine phosphokinase, 2 mg/ml, pH 7.4, with 0.1% BSA, and with AVP at a final concentration of 10^{-8} M. The reaction was terminated by the addition of 20 μl of 10% TCA, and the cAMP generated was determined by RIA after the extraction of TCA with water-saturated ether.

Each experiment consisted of more than triplicate incubations for each experimental condition using nephron segments dissected from one mouse. The mean of the cAMP values obtained in each condition was taken as one value or a single data point. In almost all experiments, the same experiments were repeated using tubules from more than four mice and the data were expressed as the mean±SE with the individual mean value from one mouse as a single data point. Data were analyzed by paired or nonpaired *t* test as appropriate, and the *P* value < 0.05 was considered significant.

Results

The results of in vitro ADP-ribosylation in dissected MCT and MAL are shown in Fig. 1. In MCT, a band at an ~ 41-kD protein was visible after a 1-h preincubation with or without pertussis toxin, and after a 3-h preincubation without pertussin toxin. This band disappeared specifically after a 3-h preincubation with pertussis toxin. The molecular weight of this band is comparable to that of the alpha subunit of inhibitory GTP binding protein G_i. In MAL, the band of the same molecular weight protein disappeared after a 6-h preincubation with pertussis toxin, but the band was very visible after a 1-h preincubation with or without pertussis toxin, and after a 6-h preincubation without pertussis toxin. These results indicate that, in our experimental condition, pertussis toxin ADP-ribosylates the alpha subunit of G_i in 3 and 6 h in MCT and MAL, respectively, and that a 1-h preincubation with pertussis toxin is not sufficient for ADP-ribosylation of the alpha subunit of G_i in these nephron segments. It should be noted that our present results of ADP-ribosylation and its modulation by pertussis toxin are rather qualitative and may not allow strict quantitative analyses. Because of a minute size of the specimens, autoradiograms were obtained after 10 d of exposure and the gels were not analyzed by isotopic counting.

Data from our and other laboratories have shown that alpha$_2$-adrenergic stimulation inhibits AVP-dependent cAMP production in MCT but not in MAL in rats (12), that 5 mM

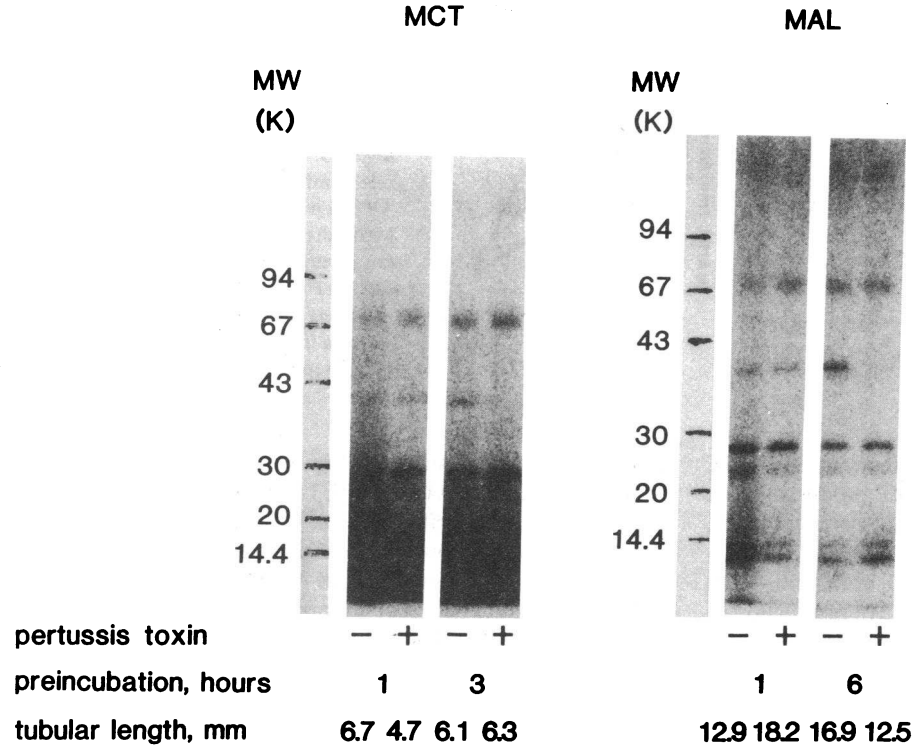

Figure 1. In vitro ADP-ribosylation in MCT and MAL. After preincubation of dissected MCT and MAL with or without pertussis toxin, tubules were permeabilized by repeated freeze-thawing in hypotonic solution and then incubated with ^{32}P-NAD and pertussis toxin. After preincubation with pertussis toxin for 3 h in MCT and for 6 h in MAL, the band of ~ 41-kD protein specifically disappeared. Tubular length used in millimeters is depicted on the bottom. See text for details.

Ca^{2+} inhibits AVP-dependent cAMP production in MAL but not in MCT in mice (14), and that PGE_2 inhibits AVP-dependent cAMP production in MAL but not in MCT in rats (13). As shown in Figs. 2 and 3, in the mouse kidney, alpha$_2$-adrenergic stimulation inhibited AVP-dependent cAMP production in MCT but only minimally, if at all, in MAL. Both 5 mM Ca^{2+} and PGE_2 inhibited AVP-dependent cAMP production in MAL but not in MCT, results which are similar to those previously reported in rats and mice (12–14). Thus, in subsequent studies, we attempted to clarify the mechanisms underlying the inhibition of AVP-dependent cAMP production by alpha$_2$-adrenergic stimulation in MCT and by 5 mM Ca^{2+} and PGE_2 in MAL when both have been dissected from the mouse kidney.

A concentration of 10^{-6} M was chosen for epinephrine, because this concentration showed significant inhibition of AVP-dependent cAMP production in MCT (Fig. 4). Moreover, this inhibition was recovered by a specific alpha$_2$-adrenergic antagonist yohimbine, indicating that this dose of epinephrine suppresses AVP-dependent cAMP production in MCT by activating alpha$_2$-adrenergic receptors.

As shown in Fig. 5, the inhibition of AVP-dependent cAMP production by 10^{-6} M epinephrine was not observed in MCT preincubated with pertussis toxin for 3 h. The pertussis toxin treatment did not affect AVP-dependent cAMP production in the absence of epinephrine. Basal cAMP production in each condition, i.e., the sum of cAMP produced during preincubation and that produced during a 3-min incubation with MIX but without AVP, was always very low, < 10 fmol/mm. The effect of pertussis toxin is most likely due to its specific action of ADP-ribosylating the alpha subunit of G_i. Thus, after preincubation of MCT with pertussis toxin for 1 h, when the

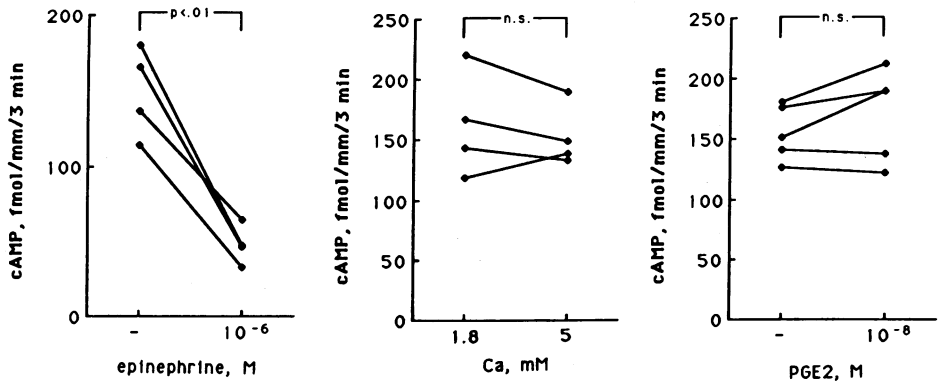

Figure 2. Effects of 10^{-6} M epinephrine, 5 mM Ca^{2+}, and 10^{-8} M PGE_2 on AVP-dependent cAMP production in MCT. After preincubation of MCT for 6 min without pertussis toxin, AVP, MIX, and test agents were added and incubated for 3 min. The effect of epinephrine was examined in the presence of 2×10^{-6} M propranolol. Each data point is the mean of values obtained from MCT of one animal and the line connecting data points indicates the results obtained in the tubules of the same animal. Epinephrine, but not high ambient Ca^{2+} nor PGE_2, inhibited AVP-dependent cAMP production in MCT.

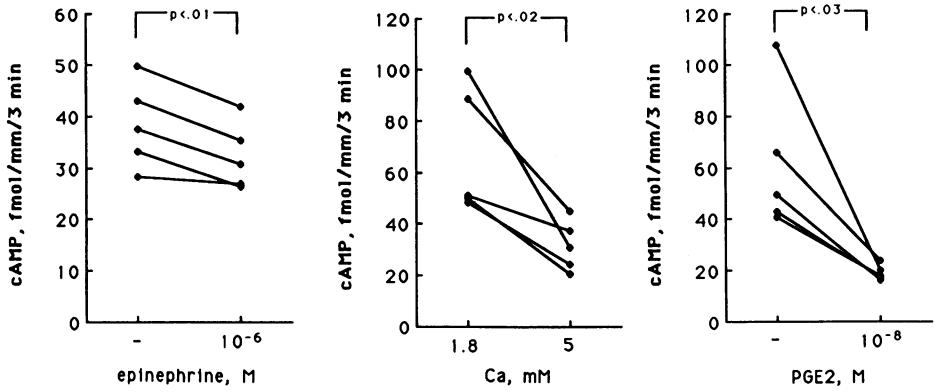

Figure 3. Effects of 10^{-6} M epinephrine, 5 mM Ca^{2+}, and 10^{-8} M PGE_2 on AVP-dependent cAMP production in MAL after a 6-min preincubation without pertussis toxin. (See the legend of Fig. 2.) High ambient Ca^{2+} and PGE_2 inhibited AVP-dependent cAMP production in MAL, but the suppression by epinephrine was trivial.

alpha subunit of G_i has not yet been ADP-ribosylated (Fig. 1), epinephrine inhibited AVP-dependent cAMP production to a similar degree in MCT preincubated without pertussis toxin, as shown in Fig. 6.

We next examined the involvement of G_i in the inhibition by high ambient Ca^{2+} of AVP-dependent cAMP production in MAL. In this set of experiments, we examined the effects of 5 mM Ca^{2+}, because this concentration of Ca^{2+} reversibly suppresses AVP-dependent cAMP production in mouse MAL (14).

As shown in Fig. 7, 5 mM Ca^{2+} in the incubation medium inhibited AVP-dependent cAMP production in MAL after a 6-h preincubation without pertussis toxin. However, the inhibition by high ambient Ca^{2+} was abolished by prior incubation of MAL with pertussis toxin for 6 h. Basal cAMP production in the absence of AVP with or without pertussis toxin treatment was very low, i.e., < 5 fmol/mm, compared with that in the presence of AVP. The effect of pertussis toxin in MAL is not due to its nonspecific action other than its specific ADP-ribosylating action of G_i, because high Ca^{2+} in the incubation medium suppressed AVP-dependent cAMP production to a similar degree after preincubation with or without pertussis toxin for 1 h as shown in Fig. 8. Interestingly, AVP-dependent cAMP production in 1.8 mM Ca^{2+} (control condition) became greater with pertussis toxin treatment for 6 h, but not for 1 h, than those without pertussis toxin treatment (Figs. 7 and 8).

Our previous data indicate that high ambient Ca^{2+}, per se, may inhibit AVP-dependent cAMP production in MAL but not in MCT (14). As shown in Fig. 9, adenylate cyclase activity of permeabilized MAL and MCT was suppressed equally by micromolar concentrations of Ca^{2+}; these results are similar to those reported previously (23). The distinct suppressive effect of high (5 mM) ambient Ca^{2+} on AVP-dependent cAMP production in intact MAL, but not in intact MCT, may be brought about through the interaction of extracellular Ca^{2+} with some machinery existing in the cell surface of MAL.

As shown in Fig. 10, PGE_2 suppressed AVP-dependent cAMP production in MAL at concentrations > 10^{-10} M as shown previously in rat MAL (13). The suppression by PGE_2 could be attenuated by pertussis toxin treatment. Thus, in MAL preincubated with pertussis toxin for 6 h, significant suppression by PGE_2 became evident at concentrations of PGE_2 10^{-9} M, but not at 10^{-10} M, as shown in Fig. 10 *a*. AVP-dependent cAMP production was significantly greater with pertussis toxin treatment than without at each concen-

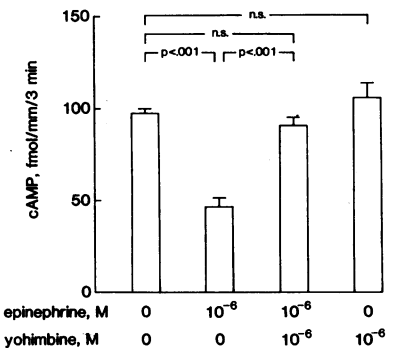

Figure 4. Effects of epinephrine and yohimbine on AVP-dependent cAMP production in MCT after preincubation without pertussis toxin for 3 h. Epinephrine inhibited AVP-dependent cAMP production in MCT, which was abolished by a specific alpha$_2$-adrenergic antagonist yohimbine. The means±SE of seven incubations of the results from a representative experiment are shown.

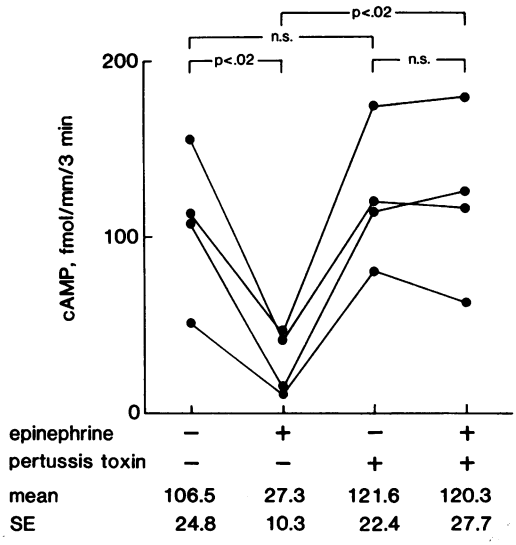

Figure 5. Effects of alpha$_2$-adrenergic agonist epinephrine, 10^{-6} M, on AVP-dependent cAMP production in MCT after preincubation with or without pertussis toxin for 3 h. Experiments were performed in the presence of 2×10^{-6} M propranolol. Each data point is the mean of values obtained from the MCT of one animal and the line connecting data points indicates the results obtained in the tubules of the same animal. Note that the inhibition by epinephrine was not observed after pertussis toxin treatment. Basal cAMP production without AVP in each condition was < 10 fmol/mm. Means±SE are given on the bottom.

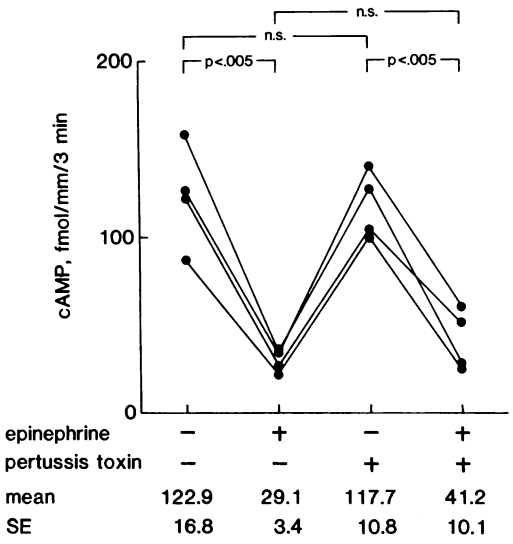

Figure 6. Effects of alpha$_2$-adrenergic agonist epinephrine on AVP-dependent cAMP production in MCT after preincubation with or without pertussis toxin for 1 h. Note that pertussis toxin had little effect on AVP-dependent cAMP production and its inhibition by epinephrine. See the legend of Fig. 5.

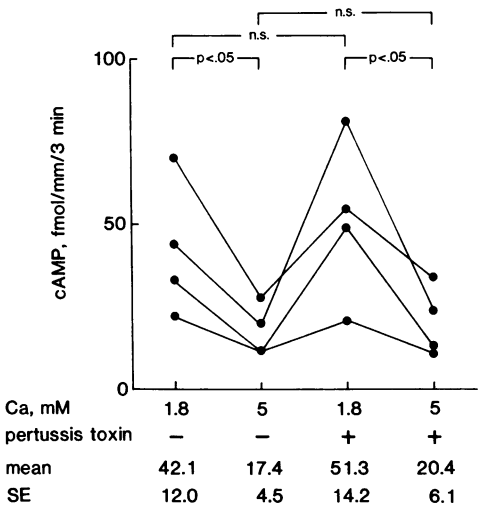

Figure 8. Effects of high ambient Ca^{2+} in the incubation medium on AVP-dependent cAMP production in MAL after preincubation with or without pertussis toxin for 1 h. Note that pertussis toxin was without effect on AVP-dependent cAMP production and its inhibition by 5 mM ambient Ca^{2+}. See the legend of Fig. 7.

tration of PGE_2 (Fig. 10 *A*). The magnitude of suppression by PGE_2 with pertussis toxin treatment was significantly less than without at each PGE_2 concentration > 10^{-10} M (Fig. 10 *B*). Thus, pertussis toxin treatment increased AVP-dependent cAMP production in each condition of PGE_2 tested and attenuated the suppression by PGE_2. As noted earlier, basal cAMP production was < 5 fmol/mm in each experimental condition. The effects of pertussis toxin in MAL, which are shown in Fig. 10, *A* and *B*, are due to its specific ADP-ribosylating action

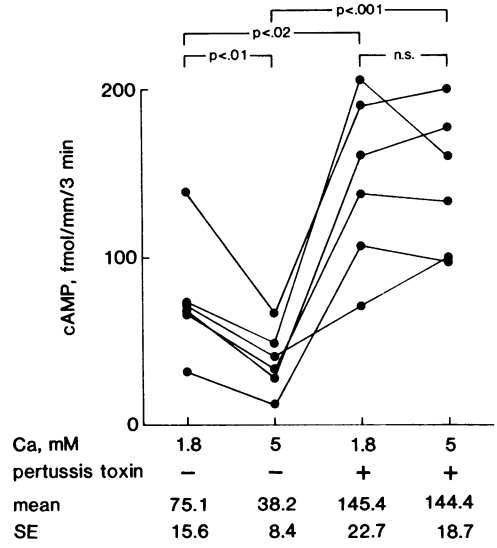

Figure 7. Effects of high ambient Ca^{2+} in the incubation medium on AVP-dependent cAMP production in MAL after preincubation with or without pertussis toxin for 6 h. Note that the inhibition by 5 mM Ca^{2+} was not observed after pertussis toxin treatment and that cAMP levels under 1.8 mM Ca^{2+} (controls) after pertussis toxin treatment were greater than those without pertussis toxin treatment. Basal cAMP production without AVP in each condition was < 5 fmol/mm. See the legend of Fig. 5.

because they were not observed with MAL preincubated with pertussis toxin for 1 h (Fig. 11).

Data shown in the previous figures were summarized in Table I so that the effects of the length of preincubation time could be evaluated. The rate of AVP-dependent cAMP production without either pertussis toxin or any inhibitory agents, and the percentage of suppression by these inhibitory agents were comparable regardless of the length of preincubation time used in the present study. Thus, the tubules remained intact with the functional adenylate cyclase regulating system even after relatively long hours of preincubation.

Discussion

AVP increases water permeability in MCT and NaCl reabsorption in MAL through cAMP production (1–8). Alpha$_2$-adrenergic agonists in MCT and high ambient Ca^{2+} and PGE_2 in MAL suppress AVP-dependent cAMP production and thereby modulate urine concentration (10–15). We attempted to clarify the mechanisms underlying the suppression of AVP-dependent cAMP production by these agents.

Our present study showed that both MCT and MAL possessed an ~ 41-kD protein that was specifically ADP-ribosylated by pertussis toxin. In the present study, preincubation with pertussis toxin of MCT for 3 h and of MAL for 6 h were needed for the full expression of the effect of pertussis toxin to ADP-ribosylate this 41-kD protein. The lengths of the preincubation time needed for ADP-ribosylation by pertussis toxin are similar to those reported for other tissues (17, 18, 21, 24).

Our present data clearly show that epinephrine suppresses AVP-dependent cAMP production in MCT, which is blocked by yohimbine, and that this suppressive action of epinephrine is also abolished by pretreatment of MCT by pertussis toxin for 3 h but not for 1 h. It has been well established by the data in other tissues that the inhibition of adenylate cyclase activity by alpha$_2$-adrenergic stimulation is mediated through the activation of G_i (16, 17). These results indicate that the same mecha-

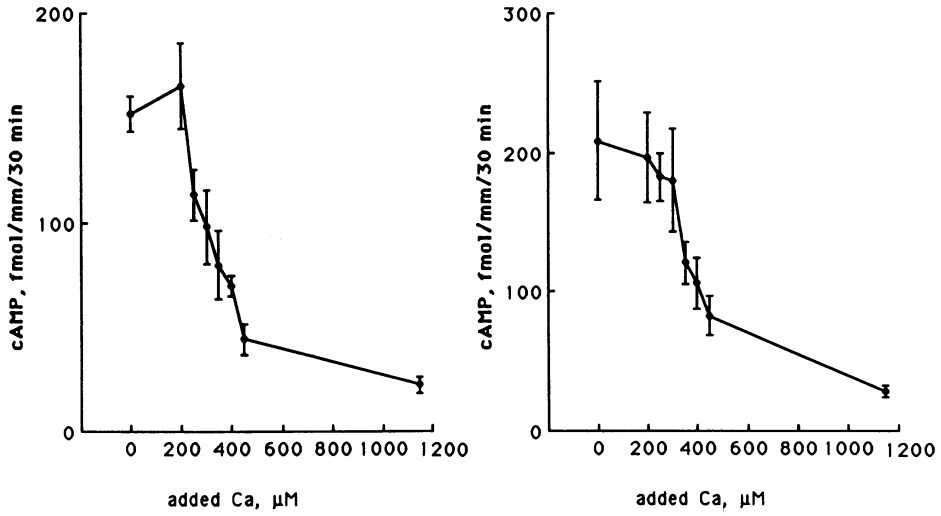

Figure 9. Effects of increasing concentration of Ca^{2+} on adenylate cyclase activity in permeabilized MAL (*left*) and MCT (*right*) in the presence of 250 μM EGTA. Results of a representative experiment are shown. Note that micromolar range of Ca^{2+} equally inhibits adenylate cyclase activity in both nephron segments. Each data point is mean±SE of five to six incubations.

nism is operating in the action of alpha$_2$-adrenergic stimulation in MCT to suppress AVP-dependent adenylate cyclase activation.

Our data also showed that the inhibition of AVP-dependent cAMP production by high ambient Ca^{2+} in MAL was abolished by pertussis toxin pretreatment of these tubular cells. The suppression of AVP-dependent cAMP production by PGE_2 in MAL was also attenuated after pertussis toxin treatment. Moreover, in MAL, but not in MCT, AVP-dependent cAMP production in the control condition with AVP, but without inhibiting agents, became greater after treatment with pertussis toxin than that without pertussis toxin. The effects of pertussis toxin are due to its specific effect of being able to ADP-ribosylate a 41-kD protein, presumably the alpha-subunit of G_i (17, 18) because the effects were not observed in MAL preincubated with pertussis toxin for only 1 h, during which time ADP-ribosylation did not occur in our experimental condition. Based on the results of our present study, it is likely that the suppression of AVP-dependent cAMP production by high ambient Ca^{2+} in MAL is mediated through the activation of G_i, and the suppression by PGE_2 in MAL is also mediated, at least in part, through the activation of G_i. Since the suppression of AVP-dependent cAMP production by PGE_2 in MAL was not completely prevented by pertussis toxin treatment in the presence of a high dose of PGE_2, it is possible that a part of the suppression by PGE_2 may be brought about through an interaction of PGE_2 with G_s as suggested by the results seen in collecting tubules (25). Moreover, our data on the reversal by pertussis toxin of the suppression of AVP-dependent cAMP production by PGE_2 in MAL corroborate the observations that PGE_2 receptors may be linked to G_i in the renal medulla (26), and that the suppression by PG of adenylate cyclase activity in adipocytes may be mediated through the activation of G_i (18).

The mechanisms by which high ambient Ca^{2+} may activate G_i and thereby suppress AVP-dependent cAMP production in

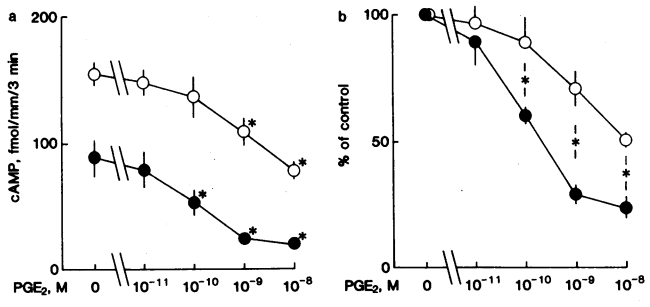

Figure 10. Effects of PGE_2 on AVP-dependent cAMP production in MAL after preincubation with or without pertussis toxin for 6 h. Results with pertussis toxin treatment are shown in open circles, and those without pertussis toxin treatment are shown in closed circles. (*a*) AVP-dependent cAMP production with and without 10^{-11}–10^{-8} M PGE_2. Basal cAMP production without AVP was < 5 fmol/mm. Each data point represents mean±SE of 5 mice. Asterisks indicate a significant difference from respective control (without PGE_2) values. (*B*) Data in *A* are expressed in percent of control to demonstrate the magnitude of suppression by PGE_2. Asterisks indicate significant differences in the magnitude of suppression.

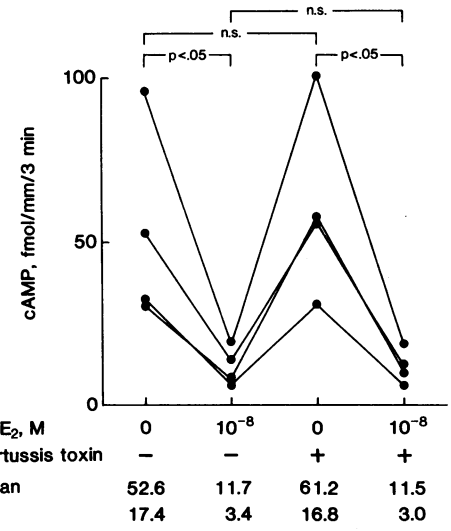

Figure 11. Effects of 10^{-8} M PGE_2 on AVP-dependent cAMP production in MAL after preincubation with or without pertussis toxin for 1 h. Note that 10^{-8} M PGE_2 suppressed AVP-dependent cAMP production to a similar degree in both conditions and that AVP-dependent cAMP production without PGE_2 in both conditions was comparable. See the legend of Fig. 5.

Table I. Effects of Preincubation Time on AVP-dependent cAMP Production in MCT and MAL

	Preincubation time		
	6 min	1 h	3 h
	fmol/mm per 3 min		
MCT			
Control	149.2±17.1	122.9±16.8	106.5±24.8
With epinephrine	47.7±7.4	29.1±3.4	27.3±10.3
% Suppression	67.5±5.6	76.0±1.2	75.4±6.4
No. of mice	4	4	4
	6 min	1 h	6 h
MAL			
Control	67.4±12.3	42.1±12.0	75.1±15.6
With 5 mM Ca	31.7±4.9	17.4±4.5	38.2±8.4
% Suppression	50.6±7.7	57.3±4.3	50.2±4.7
No. of mice	5	4	6
Control	64.3±12.4	52.6±17.4	88.5±14.6
With PGE_2	19.1±1.5	11.7±3.4	19.3±2.6
% suppression	65.5±4.8	77.3±2.0	76.3±4.5
No. of mice	5	4	5

Cyclic AMP production (fmol/mm per 3 min) and percent suppression (%) by epinephrine (10^{-6} M) in MCT and by high ambient Ca^{2+} (5 mM) and PGE_2 (10^{-8} M) are shown. Note that cAMP production in the absence of any inhibitors (controls) and percent suppression by the inhibitors were comparable regardless of the length of preincubation time. Isolated tubules were preincubated without pertussis toxin. Values are the mean±SE and the numbers of mice for each experiment are given at the bottom of each set of experiment.

MAL is not clear. As might be expected from looking at the available data (23), adenylate cyclase activation by AVP in permeabilized tubular cell preparations were inhibited equally in both MCT and MAL by a micromolar concentration of Ca^{2+} (Fig. 9). By contrast, inhibition by high ambient Ca^{2+} of cAMP production in intact cell preparations was seen only in MAL and not in MCT. These results suggest that high extracellular Ca^{2+} suppresses AVP-dependent cAMP production in MAL, probably through the interaction of extracellular Ca^{2+} with some machinery existing on the plasma membranes of this nephron segment. It is also indicated that this mechanism is sensitive to pertussis toxin. Thus, it may be possible that MAL possesses a Ca^{2+} receptor on the cell surface that is linked to G_i and is similar to those proposed in parathyroid cells (27, 28). These possibilities are only speculative and need further study.

It is of note that AVP-dependent cAMP production in MAL, but not in MCT, became greater after 6 h of preincubation with pertussis toxin. These data may suggest the possibility that G_i is relatively dominant over G_s in MAL, which may have some functional significance in the hormonal regulation of MAL (29). Alternately, it is possible that AVP-sensitive cAMP production without PGE_2 or high ambient Ca^{2+} was partially suppressed via G_i activation by the 1.8 mM Ca^{2+} present in the control incubation medium, and that pertussis toxin prevented this G_i activation under control conditions.

Despite relatively long hours of preincubation, we believe that the tubules remained intact with the functional adenylate cyclase regulating system for the following reasons. First, as shown in the Results, the rates of AVP-dependent cAMP production without pertussis toxin or any inhibitory agents are comparable in both nephron segments preincubated for varied lengths of time (Table I). This indicates that tubules possess an intact adenylate cyclase-stimulating system even after long hours (3–6 h) of preincubation. Second, the magnitudes of suppression by epinephrine in MCT and by high ambient Ca^{2+} and PGE_2 in MAL without pertussis toxin are also comparable regardless of the length of preincubation time (Table I), indicating that adenylate cyclase-inhibiting systems sensitive to these agents also remain intact during the preincubation condition employed in the present study.

In summary, our present study showed that ADP-ribosylation of a 41-kD protein by pertussis toxin abolished or attenuated the inhibition of AVP-dependent cAMP production by $alpha_2$-adrenergic stimulation in MCT and by high ambient Ca^{2+} and PGE_2 in MAL. Moreover, pertussis toxin pretreatment resulted in an increase in AVP-dependent cAMP production in the absence of these inhibitory agents in MAL but not in MCT. These data suggest that the inhibition of AVP-dependent cAMP production by $alpha_2$-adrenergic stimulation in MCT, and by high ambient Ca^{2+} and PGE_2 in MAL are, at least in part, mediated through the activation of G_i.

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