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Forskolin Increases Osmotic Water Permeability of Rabbit Cortical Collecting Tubule

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Summary. Forskolin is a unique diterpene that may directly activate the catalytic subunit of adenylate cyclase. We therefore examined the effect of 50 μM forskolin on osmotic water permeability in rabbit cortical collecting tubules perfused *in vitro*. Forskolin increased net volume flux (J_v, from 0.30 to 1.22 nl/mm/min, $P < 0.02$) in all tubules. The hydro-osmotic effect of forskolin was similar with respect to magnitude and time course to that produced by a maximal dose (250 μU/ml) of arginine vasopressin. An additive effect on J_v and L_p was not observed when maximal concentrations of forskolin and arginine vasopressin were given simultaneously. The compound $d(CH_2)_5$Tyr(Et) VAVP, which noncompetitively inhibits the vasopressin receptor, significantly reduced collecting tubular hydro-osmotic response to arginine vasopressin. In contrast, the hydro-osmotic response to forskolin was maintained in the presence of $d(CH_2)_5$ Tyr(Et)VAVP. However, the hydro-osmotic response to forskolin could be inhibited by 1.0 μM guanine 5′-(β,γ-imido) triphosphate (GppNHp) and by the calmodulin inhibitor N-(6-amenohexyl)-5-chloro-1-naphthalenesulfonamide (W-7). These results demonstrate that forskolin exerts an hydro-osmotic effect in the mammalian nephron which occurs independent of the vasopressin receptor. Guanine nucleotide regulatory proteins may modulate the osmotic water permeability effect of forskolin. Finally, calmodulin is required for full expression of the effect of forskolin to increase osmotic water flux.

Key Words forskolin · collecting tubule · adenylate cyclase · water permeability · vasopressin

Introduction

Forskolin is a unique diterpene compound found in the root of the coleus plant (Seamon & Daly, 1981*a*; Seamon, Padgett & Daly, 1981). Forskolin activates adenylate cyclase with subsequent generation of intracellular cyclic 3′,5′ adenosine monophosphate (cAMP) in numerous eukaryotic cells and in intact tissues (Seamon & Daly, 1981*a*). Based on biochemical studies, forskolin appears to act, at least in part, by activating directly the catalytic subunit of adenylate cyclase. Despite substantial biochemical studies on the effect of forskolin, there is little information on the effect of forskolin on membrane transport. We therefore examined the effect of forskolin on basal and arginine vasopressin (AVP) stimulated osmotic water permeability and hydraulic conductivity in rabbit cortical collecting tubules perfused *in vitro*.

Materials and Methods

Perfusion of collecting tubules was carried out by slight modifications of the method developed by Burg (Burg, Grantham, Abramow & Orloff, 1966; Horster & Zink, 1982). New Zealand white rabbits weighing 1–2 kg were maintained on rabbit chow (Purina, St. Louis, MO) and *ad libitum* water. The animals were killed by cervical dislocation, and the left kidney was quickly removed. One to two mm slices were cut along the cortico-medullary axis and placed in a glass dish in a solution of NaCl 115, $MgSO_4$ 1.2, $CaCl_2$ 1.0, KCl 5.0, sodium acetate 10, NaH_2PO_4 1.2, Na HCO_3 25, and dextrose 5.5 (all in mmol/liter) with 5% bovine serum albumin. This solution was maintained at 4°C and pH of 7.4 during dissection. The cortex was separated from outer medulla and cortical collecting tubules (CCT) were dissected from medullary rays. Collecting tubules were identified by the presence of branching, straight configuration, light granular appearance with fuzzy borders, and generally open lumens. Tubules were taken distal to the last branch. Tubules were transferred to a Lucite perfusion chamber (volume about 2.0 ml) set on the stage of an inverted phase contrast microscope (Olympus, Model CK, Tokyo, Japan).

Custom capillary glass (Drummond Scientific Co.) was used to make (Stoelting Microforge, Chicago, IL) pipettes of required dimensions. Tubules were aspirated into the tip of a holding pipette (OD about 32–36 μm) up to constriction of 16–18 μm. A perfusion pipette (about 12–14 μm) was advanced into the lumen and extended 50–100 μm beyond the tip of the holding pipette. A large outer pipette, containing Sylgard 184, was advanced so that Sylgard covered the holding pipette and tubule up to the tip of the perfusion pipette. Tubules were usually perfused within 30 min of kidney removal. A fluid exchange pipette inside the perfusion pipette was used as a means of changing perfusion fluid. The free end of the tubule was aspirated into a collection pipette, which had an inner diameter slightly less than the outer diameter of the tubule. The tip of the pipette was filled with Sylgard 184,

and the tubule was aspirated through the Sylgard. Water-equilibrated mineral oil was introduced into the collection pipette, and a calibrated volumetric pipette (80 – 110 nl) was used to obtain precisely timed samples of constant volume. Samples were separated by water-equilibrated mineral oil.

Tubules were bathed in the same solution used for dissection except that no albumin was present. Bath fluid of pH 7.40 and temperature of 25°C was completely changed every 4–5 min. All studies were carried out at 25°C because the hydro-osmotic response to vasopressin appears to be more stable at this temperature (Hall & Grantham, 1980). Tubules were perfused at rates of 5–15 nl/min by adjusting hydostatic pressure of the fluid entering the fluid exchange pipette. Perfusion fluid composition was the same as bathing fluid except that the final concentration of NaCl was reduced to 50 mmol/liter. Perfusion fluid also contained a sufficient amount of ^{14}C-inulin (New England Nuclear, Boston, MA) to result in collected fluid cpm at least 10-fold above background. The ^{14}C inulin was dissolved in perfusion solution and passed through a 0.22-μm filter prior to use. The tubule was visually inspected at 1–3 min intervals throughout the study. Tubular length was measured at the conclusion of each study by a calibrated microscope eyepiece reticle.

Collected samples were placed under water-equilibrated mineral oil on the bottom of a siliconized dish. Approximately 50-nl aliquots of these samples were taken for scintillation counting (Packard Tricab 460C) and 10–20 nl for osmolality measurements (Clifton Nanoliter Osmometer, Bedford, MA).

Arginine vasopressin (AVP) was obtained from Parke Davis (Detroit, MI) in the form of pitressin. This was diluted in bathing solution and frozen at −4°C until use in a final concentration of 250 μU/ml. d(CH$_2$)$_5$Tyr(Et)VAVP was a generous gift of M. Manning, University of Toledo, and was dissolved and administered at 2.5×10^{-7} M in the bathing fluid. Forskolin was purchased from Calbiochem and stored at described by Seamon and Daly (1981*a*). Guanosine 5′-(β,γ-imido) triphosphate (GppNHp) was obtained from Sigma (St. Louis, MO) and dissolved in bathing fluid.

Perfusion rate was calculated by the rate of appearance of the impermeant marker ^{14}C-inulin in the collection pipette according to the algorithm:

$$\text{Perfusion rate} = \frac{[^{14}C]c}{[^{14}C]p} \times \text{collection rate}$$

where $[^{14}C]c$ = cpm of collected fluid and

$[^{14}C]p$ = cpm of perfusate.

Collection rate was measured directed with a calibrated volumetric pipette and stopwatch. Net volume of water reabsorption (J_v, nl/mm/min) was calculated as perfusion rate minus collection rate divided by length.

Hydraulic conductivity was calculated from the formula:

$$L_p = \frac{V_o C_o}{RTA}\left[\frac{C_o - C_L}{C_o C_L C_b} + \frac{1}{(C_b)^2} \ln \frac{(C_L - C_b)C_o}{(C_o - C_b)C_L}\right]$$

where L_p is in cm/atm sec $\times 10^{-7}$, V_o is the perfusion rate in cm^3/sec; C_o, C_L, and C_b are the osmotic concentrations of perfusate, collected fluid, and bathing medium, respectively; R is the gas constant, T is the absolute temperature, and A is the surface area (calculated from the length of the tubule and an assumed internal diameter of 20 μm). Collected fluid osmolality was calculated from measured perfusate osmolality and the relative increase in the concentration of the volume marker measured in the collected fluid.

Seven groups of experiments were performed. Each study began 120–200 min following initiation of tubular perfusion when distinct intercellular spaces were no longer visible. At this time, three to four control collections were obtained over 30–40 min. After this interval, the following agents were placed in bathing fluid: Group 1, AVP (250 μU/ml); Group 2, forskolin (50 μM); and Group 3, AVP (250 μU/ml) and forskolin (50 μM). Collections were then made for 30–60 min. In groups 4 and 5, after initial control collections were made, VAVP (2.4×10^{-7} M) was added to bathing fluid and an additional 4–5 collections over 30–40 min were made. Then, either AVP (250 μU/ml, group 4) or forskolin (50 μM, group 5) were added to bathing fluid and an additional 4–5 collections were obtained. In group 6, the effect of 1.0 μM GppNHp on basal and forskolin-stimulated osmotic water permeability was examined. In these studies, after initial control samples were obtained, 1.0 μM GppNHp was added to the bathing fluid. Collections were then made over the next 30 min at which time forskolin (50 μM) was also added to the bathing fluid ($n = 5$). An additional 4–5 collections were made after combined forskolin and 1.0 μM GppNHp. Additional studies were carried out in seven tubules to determine if the acute administration of GppNHp affects an established hydro-osmotic response to forskolin. In these studies, after basal collections were made, forskolin (50 μM) was added to bathing fluid and an additional 3–4 collections obtained. Then, either GppNHp ($n = 3$) or GppNHp carrier ($n = 4$) was added to the forskolin in the bathing fluid and an additional four collections made. In an additional four tubules, we examined the effect of 1.0 μM GppNHp on the hydro-osmotic response to 250 μU/ml AVP. Collections were made before and sequentially following AVP in tubules bathed in 1.0 μM GppNHp. In group 7, we examined the effect of an inhibitor of calmodulin-dependent protein phosphorylation (W-7, Nishikawa & Hidaka, 1982; Tanaka, Ohmura & Hidaka, 1982*a*; Tanaka, Ohmura, Yamakado & Hidaka, 1982*b*) on the osmotic water permeability response to forskolin. In these studies, collections were made before and sequentially following 50 μM forskolin in tubules pretreated (30 min) with 30 μM W-7.

Statistics

The results were analyzed by either unpaired Student's t test or Duncan's method of multiple comparisons.

Results

The number of tubules in each group, mean tubular length, mean perfusion rate, and mean basal and peak J_v values for groups 1 through 7 are depicted in the Table. There were no significant differences when tubular lengths and mean perfusion rates were compared in the six groups. A striking effect of both AVP (group 1) and forskolin (group 2) to increase J_v (mean values in the Table) and L_p (Fig. 1) is apparent. Sequential measurements of mean net volume flux are depicted in Fig. 2. The time course and magnitude of effect of AVP and forskolin to increase osmotic water permeability were similar. In

addition, both AVP and forskolin resulted in cell swelling and marked accentuation of intercellular spaces within 5–8 min of administration. In additional studies (n = 3), the hydro-osmotic effect of 100 μM forskolin was examined and was not greater than that observed with 50 μM forskolin, suggesting that 50 μM forskolin is a maximal dose. To determine if the hydro-osmotic effects of VP and forskolin were additive, both agents were administered simultaneously. The peak J_v and L_p values when AVP and forskolin were given together did not differ from values obtained with either agent alone (Fig. 1; Table, group 3). To insure that forskolin did not exert a nonspecific toxic effect to increase water permeability, J_v responses to forskolin were carried out in cortical thick ascending limb of Henle, another water impermeable rabbit nephron segment. In these studies (n = 3) J_v and L_p values did not differ from zero through the entire study.

The effect of VAVP on basal, AVP, and forskolin-stimulated osmotic water permeability was next examined. In these studies, AVP and forskolin were given 30–40 min after administration of VAVP. This time interval was selected because, as demonstrated in Fig. 3, VAVP resulted initially in a transient increase in J_v and L_p ($70 \pm 10 \times 10^{-7}$ cm/atm sec increment, $P < 0.05$). However, at the time of administration of AVP and forskolin, J_v and L_p had returned to basal levels. Moreover, in three tubules, J_v and L_p remained constant and not different from zero from 60 min following the initial transient agonistic response. Pretreatment with VAVP significantly reduced the osmotic water permeability responses to 250 μU/ml AVP (n = 6) (Table, Figs. 1, 2). In contrast, J_v and L_p responses to forskolin were not lowered by VAVP (Figs. 1 and 2, Table).

In liver membranes and adipocytes, low dose (<1.0 μM) GppNHp inhibits the effect of forskolin (50 μM) to stimulate adenylate cyclase (Seamon & Daly, 1982; Hudson & Fain, 1983). We therefore examined the effect of 1.0 μM GppNHp on basal and forskolin-stimulated J_v and L_p (Table, Group 6). In these studies, 1.0 μM GppNHp did not alter J_v from zero (n = 6, 0.04 ± 0.10 before to 0.07 ± 0.05 nl/mm/min after). However, 1.0 μM GppNHp pretreatment (n = 5) attenuated the effect of 50 μM forskolin to increase J_v (Fig. 4). Thus, peak J_v after

Table. Number, length, perfusion rate, and mean basal and maximal stimulated J_v in tubules from groups 1 through 7

Group	Tubular length (mm)	Perfusion rate (nl/min)	Mean basal J_v (nl/mm/min)	Mean peak J_v (nl/mm/min)
1. AVP (n = 7)				
Mean	1.11	10.2	0.26	1.11[a]
SEM	0.20	1.2	0.15	0.27
2. Forskolin (n = 6)				
Mean	1.04	9.3	0.30	1.22[a]
SEM	0.12	0.6	0.14	0.18
3. Forskolin + AVP (n = 5)				
Mean	1.26	10.0	0.24	0.98[a]
SEM	0.25	0.8	0.16	0.20
4. AVP + VAVP (n = 6)				
Mean	1.01	9.2	0.20	0.43
SEM	0.26	0.5	0.10	0.25
5. Forskolin + VAVP (n = 5)				
Mean	1.37	10.4	0.20	1.37[a]
SEM	0.24	0.7	0.12	0.18
6. Forskolin + GppNHp (n = 5)				
Mean	1.48	10.2	0.10	0.18
SEM	0.30	0.8	0.10	0.15
7. Forskolin + W-7 (n = 5)				
Mean	1.56	10.5	0.30	0.60[a]
SEM	±0.30	±1.0	±0.18	±0.22

[a] $P < 0.05$.

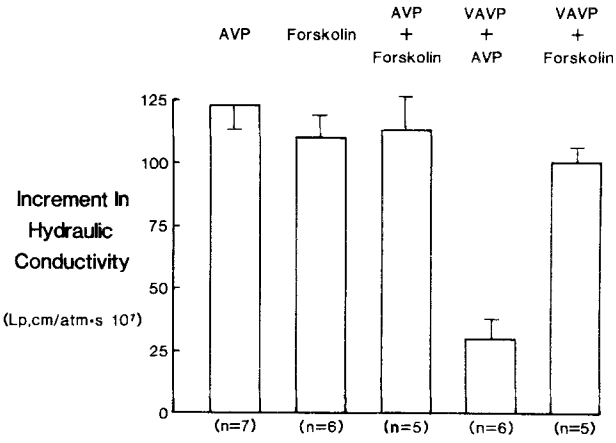

Fig. 1. Increment in hydraulic conductivity in groups 1–5

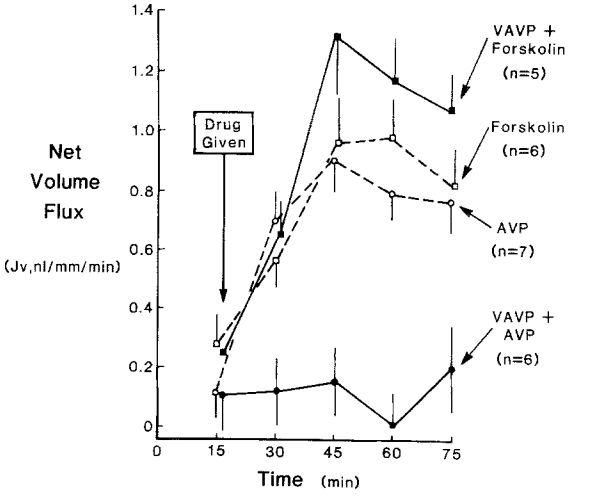

Fig. 2. Sequential measurements of J_v in groups 1, 2, 4 and 5. Group 1 (AVP alone) is denoted by open circles and broken lines, group 2 (forskolin alone) by open squares and broken lines, group 4 (VAVP plus AVP) by solid circles and lines, and group 5 (VAVP plus forskolin) by solid squares and lines

forskolin in the absence (1.22 ± 0.18 nl/mm/min) and in the presence of GppNHp (0.18 ± 0.15 nl/mm/min) differed significantly ($P < 0.01$). The increment in L_p with forskolin alone was 110 ± 16 × 10^{-7} ctm/atm · sec and with GppNHp pretreatment then forskolin, 25 ± 15 × 10^{-7} ctm/atm · sec ($P < 0.01$). In addition, when 1.0 μM GppNHp was added 30 min after forskolin, a rapid decline in J_v (0.93 ± 0.20 to 0.22 ± 0.20 nl/mm/min, $n = 3$) occurred over the next 30 min. In contrast, in the tubules with forskolin alone studied alone over the same time interval J_v decreased significantly ($P < 0.05$) less (0.91 ± 0.21 to 0.71 ± 0.22 nl/mm/min, $n = 4$). We also undertook studies on the effect of GppNHp on AVP effect. In four tubules pretreated with 1.0 μM GppNHp, mean J_v before was 0.14 nl/mm/min before 250 μU/ml AVP. Mean J_v's at 10 min intervals following AVP were 0.30 ± 0.07, 0.52 ± 0.2, 0.81 ± 0.15, and 0.81 ± 0.13 nl/mm/min. The first two values are significantly lower ($P < 0.025$) than with AVP alone.

As can be observed in the Table (group 7 and in Fig. 5), W-7 pretreatment also completely inhibited the hydro-osmotic effect of 50 μM forskolin.

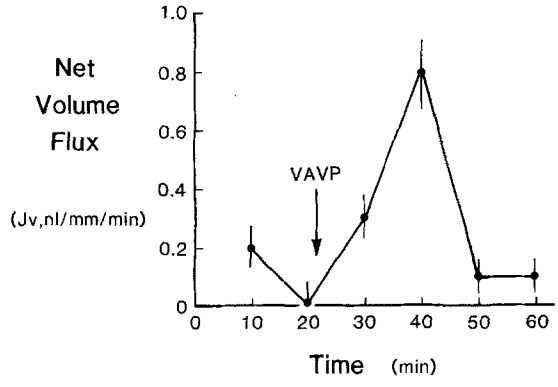

Fig. 3. Effect of VAVP on J_v

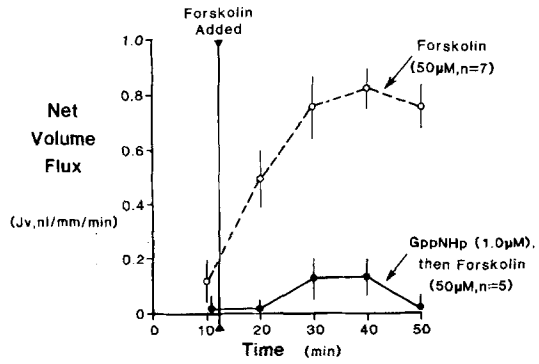

Fig. 4. Effect of pretreatment with 1.0 μM GppNHp on osmotic water permeability response to forskolin

Discussion

The adenylate cyclase system is composed of at least three distinct protein components including a specific hormone receptor, guanine nucleotide regulatory proteins capable of modulating receptor input, and a catalytic subunit, which converts the substrate magnesium adenosine triphosphate to 3',5' cyclic adenosine monophosphate (cAMP). Forskolin has been described to activate soluble adenylate cyclase (Seamon & Daly, 1981*a*; Seamon et al., 1981) and to activate adenylate cyclase in mutant cells that do not contain functional stimulatory guanine nucleotide regulatory proteins (Seamon & Daly, 1981*b*; Clark et al., 1982; Downs & Aurbach, 1982; Hildebrandt, Honoue & Birnbaumer, 1982; Awad, Johnson, Jakobs & Schultz, 1983). Thus, based on biochemical studies, forskolin appears to act at least in part by directly activating the catalytic subunit of adenylate cyclase.

Although the biochemical effects of forskolin are well documented, there is little information on the effects of forskolin on membrane transport processes. In amphibian skin and choroid plexus and in rat colon, three membranes that react to exogenous cAMP, forskolin exerted a dose-dependent increase in short-circuit current (Cuthbert & Spayne, 1982; Saito & Wright, 1983). In salivary glands of the blowfly, forskolin stimulated cAMP production and increased transepithelial movement of calcium (Litosch, Saito & Fain, 1982). In a preliminary report, forskolin exerted a dose-dependent effect to increase osmotic water and urea permeability and cell cAMP content in toad urinary bladder (Arenare & Forrest, 1983). In the present studies, 50 μM forskolin resulted in a marked increase in osmotic water flux and hydraulic conductivity of the rabbit cortical collecting tubule. The hydro-osmotic effect of forskolin paralleled that of AVP with regard to mag-

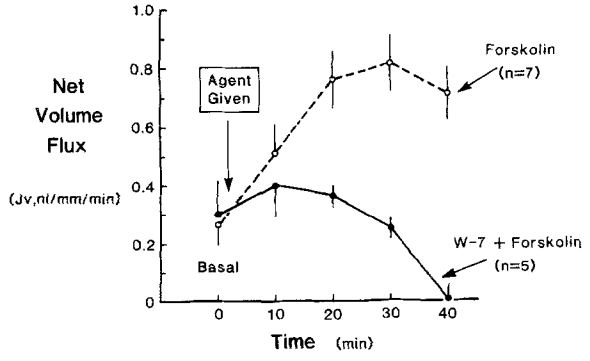

Fig. 5. W-7 decreases forskolin response. Effect of pretreatment with 30 μM W-7 on osmotic water permeability response to forskolin

nitude and time course (Fig. 2). This effect could not be attributed to nonspecific toxic injury since tubular morphology appeared intact. In addition, forskolin did not alter J_v from zero in perfused cortical thick ascending limb of Henle, another water impermeable segment of the rabbit nephron that does not contain large amounts of AVP-stimulated adenylate cyclase. Since cAMP is clearly the second messenger of AVP effect, the present and previous studies demonstrate the feasibility of using forskolin as a pharmacologic probe to study the physiologic effects of adenylate cyclase activation in rabbit collecting tubule.

In biochemical studies, forskolin stimulates solubilized, membrane-free adenylate cyclase (Seamon & Daly, 1981*a*). These observations suggest that forskolin can act independent of hormone receptor input. We therefore examined the effect of VAVP on the hydro-osmotic action of forskolin. In previous studies, VAVP inhibited AVP-stimulated adenylate cyclase in rat cortical and medullary collecting tubule and medullary thick ascending limb but did not alter adenylate cyclase stimulated by parathyroid hormone in cortical ascending limb, by glucagon in medullary thick ascending limb, and by calcitonin in cortical collecting tubule. In addition, VAVP inhibited binding of ^{3}H-AVP to membranes from rat medullary collecting tubules. The kinetics of this inhibition suggest a noncompetitive type of inhibition (Kim, Dillingham & Summers, 1984). In the present studies, VAVP exerted an initial transient effect to increase J_v (Fig. 3). A similar initial, transient agonist effect of VAVP occurs in rats *in vivo* (Ishikawa & Schrier, 1982; Ishikawa, Kim & Schrier, 1983). Despite subsequent clear-cut (90%) inhibition of effect of AVP on J_v and L_p, VAVP did not inhibit the effect of forskolin to increase J_v and L_p. These physiologic observations support that the hydro-osmotic effect of forskolin occurs independent of the AVP receptor.

In many membrane systems including rat cerebral cortex, rat adipose, murine lymphoma cells, and human platelets, forskolin potentiates adenylate cyclase responses to hormones such as norepinephrine, isoproterenol, prostaglandin E_2, and vasoactive intestinal peptide (Seamon et al., 1981; Clark et al., 1982; Fradkin, Cook, Kilhoffer & Wolff, 1982; Siegel, Daly & Smith, 1982). Moreover, preliminary observations by Arenare and Forrest (1983) demonstrate that maximal doses of forskolin and AVP exert synergistic effects to increase osmotic water permeability in toad urinary bladder. A synergistic effect of forskolin and agonist hormones suggest that forskolin may potentiate receptor input by allosteric activation of adenylate cyclase. In the present studies, maximal doses of AVP and forskolin did not exert an additive physiologic effect. The doses of forskolin and AVP utilized in the present study were clearly additive with regard to osmotic water permeability in toad urinary bladder (Arenare & Forrest, 1983). It is possible that a synergistic effect on osmotic water permeability would be observed with use of submaximal doses of AVP and forskolin. Thus, further studies are necessary to clarify the interaction between AVP and forskolin in the rabbit collecting tubule.

In several cyclic nucleotide systems, the catalytic subunit of adenylate cyclase is subject to dual regulation by both inhibitory and stimulatory guanine nucleotide regulatory proteins (Seamon & Daly, 1982; Cooper, 1983; Jakobs & Schultz, 1983). One characteristic of such dually regulated systems is inhibition of adenylate cyclase at low (<1.0 μM) concentrations of GppNHp (Seamon & Daly, 1982). In the present studies, 1.0 μM GppNHp inhibited the effect of forskolin to both initiate and maintain an increase in J_v. An inhibitory effect of 1.0 μM GppNHp on forskolin-stimulated adenylate cyclase has been observed in biochemical studies in several mammalian membranes (Seamon & Daly, 1982; Hudson & Fain, 1983). We also observed that 1.0 μM GppNHp significantly attenuated the early hydro-osmotic response to AVP. However, maximal AVP response was not lowered. Thus, the effects of GppNHp on osmotic water flux response to AVP and forskolin differed. It is possible that AVP *per se* inactivates inhibitory or activates stimulatory guanine nucleotide regulatory proteins. Thus, further studies including biochemical analysis will be required to elucidate the interaction of GppNHp with forskolin and AVP. However, the present physiologic and previous biochemical studies (Hildebrandt et al., 1982; Hudson & Fain, 1983; Seamon & Daly, 1982) suggest that guanine nucleotide regulatory proteins are capable of an inhibitory influence on the action of forskolin.

In the present studies, W-7, an inhibitor of calmodulin-mediated protein phosphorylation (Tanaka, 1982*a*,*b*) inhibited the hydro-osmotic response to forskolin. Studies on toad urinary bladder have demonstrated that trifluoperazine, another calmodulin inhibitor, impairs maximal osmotic water flux response to AVP and cAMP (Levine, Kachadorian, Levin & Schlondorff, 1981). Together, the present and previous studies suggest that calmodulin is necessary for full expression of the osmotic water permeability response to forskolin.

In summary, forskolin exerts an effect to increase osmotic water flux and hydraulic conductivity in the mammalian collecting tubule. The hydro-

osmotic effect of forskolin parallels that of AVP, is not additive to that of AVP in maximal doses, and occurs in the presence of noncompetitive inhibition of the AVP receptor. The hydro-osmotic effect of forskolin can be attenuated by 1.0 μM GppNHp and W-7. Forskolin may be useful as a pharmacologic probe in states associated with an impaired hydro-osmotic response to AVP. In such a setting, an intact response to forskolin suggests either the AVP receptor or guanine nucleotide binding proteins as the site of the defect.

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