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AUGMENTATION OF FORSKOLIN-INDUCED cAMP PRODUCTION BY OUABAIN IN RAT RENAL PAPILLARY COLLECTING TUBULE CELLS IN CULTURE

San-e Ishikawa and Toshikazu Saito

Division of Endocrinology and Metabolism, Department of Medicine, Jichi Medical School, Tochigi 329-04, Japan

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SUMMARY: The effect of extracellular calcium (Ca^{2+}) on the cellular action of forskolin was studied using a Na^+, K^+-ATPase inhibitor ouabain in rat renal papillary collecting tubule cells in culture. Forskolin-induced cAMP production was enhanced by the pretreatment of cells with ouabain, providing that a dose-dependent curve with forskolin shifted to the left. The enhancement by ouabain of cellular cAMP production in response to forskolin was totally blunted by cotreatment with cobalt, verapamil, or Ca^{2+}-free medium containing 1 mM EGTA. In addition, two dissimilar antagonists of calmodulin, namely trifluoperazine and N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W - 7), attenuated the ouabain's effect on cAMP production in response to forskolin. These results therefore indicate that ouabain enhances the activation of adenylate cyclase by forskolin, mediated through cellular free Ca^{2+}, in renal papillary collecting tubule cells, and that extracellular Ca^{2+} is an important source for cellular Ca^{2+} mobilization by ouabain.

We have demonstrated that arginine vasopressin (AVP) and forskolin increase cellular adenosine-3', 5'-cyclic monophosphate (cAMP) production in renal papillary collecting tubule cells (1). Calcium (Ca^{2+})-binding protein calmodulin modulates the cellular action of AVP and forskolin to produce cAMP (1). Forskolin is a well-known diterpene activator of adenylate cyclase, and thus the site of action of calmodulin is probably the catalytic unit.

Biochemical and physiological studies have shown that Ca^{2+} modulates the cellular action of AVP in renal collecting tubule (2 - 5). There are optimal concentrations of extracellular Ca^{2+} and intracellular Ca^{2+} for the AVP-induced cAMP production (6 - 7). In this point of view, ouabain enhanced the celular action of AVP, as 1×10^{-5} M ouabain increased cellular free Ca^{2+} concentration ($[Ca^{2+}]i$) to approximately 142 nM (mean value) which was in the range of its optimal concentration (8). When Na^+, K^+-ATPase is blocked by ouabain, the intracellular Na^+ concentration is raised. In this state, Na^+/Ca^{2+} exchange can move in a direction opposite to that of Na^+ electrochemical gradient (9).

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The present study used ouabain in rat renal papillary collecting tubule cells to determine if cellular Ca^{2+} mobilization modulates the effect of forskolin. Whether extracellular Ca^{2+} is an important source of Ca^{2+} for the cellular action of forskolin was also examined.

MATERIALS AND METHODS

Cell culture

The experimental procedure was similar to that in our previous studies (1, 10), modified from the study of Grenier et al (11). Eight male Sprague-Dawley rats weighing 150 - 175 g were used. Under sterile conditions renal papillary tissues were dissected out and minced with 1 ml collagenase (1 mg/ml; type II, Worthington Biochemicals, Freehold, N. J.) using a sharp blade. Collagenase was dissolved in Krebs-Ringer buffered saline (pH 7.4). They were transferred into culture tubes containing 3 ml collagenase (1 mg/ml) and kept at 37 C for 90 min. The minced papillary tissues were exposed to hypotonic solution, and 10 ml 10% bovine serum albumin (Sigma, St. Louis, MO) dissolved in Krebs-Ringer buffered saline. After centrifugation, the pellets were resuspended with Dulbecco's modified Eagle's minimal essential medium (DMEM; Flow Laboratories, McLean, VA) supplemented with 10% fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 ug/ml); the osmolality was adjusted to 600 mOsm/kg H_2O by the addition of NaCl and urea in a molar ratio of 1 : 2 (10). The dispersed cells and tubular fragments were plated in a total of 108 wells of 24-well tissue culture clusters (Costar, Cambridge, MA) with 1 ml DMEM containing 10% fetal bovine serum, penicillin and streptomycin and kept at 37 C in a humidified incubator under 95% air and 5% CO_2. On day 2 of culture the medium was changed to 1 ml 99% DMEM containing 1% fetal bovine serum, 5 ug/ml insulin (Sigma), 5 ug/ml transferrin (Sigma), 5×10^{-8} M hydrocortisone (Calbiochem-Boehringer, La Jolla, CA), 5×10^{-8} M T_3 (Sigma), 100 U/ml penicillin, and 100 ug/ml streptomycin, with an osmolality of 600 mOsm/kg H_2O as described above. The culture cells were studied on day 5 of culture.

Cellular cAMP Production

The experimental procedure was similar to our previous studies (1, 10). The cells were rinsed twice with 1 ml Krebs-Ringer buffered saline, which osmolality was 600 mOsm/kg H_2O, produced by adding NaCl and urea in a molar ratio of 1 : 2. They were preincubated for at least 1 h with ouabain (Sigma), cobalt (Co^{2+}), verapamil (Eizai Pharmaceutical Co., Tokyo, Japan), N-(6-aminohexyl)-5-chloro-1-naphthalanesulfonamide (W-7; Seikagaku Kogyo, Tokyo, Japan), trifluoperazine (Yoshitomi Pharmaceutical Co., Osaka, Japan) or Ca^{2+}-free medium containing 1×10^{-3} M EGTA. Ouabain is an inhibitor of Na^+, K^+-ATPase. Co^{2+} is an inorganic Ca^{2+} channel blocker, and verapamil is a blocker of cellular Ca^{2+} uptake. Trifluoperazine and W-7 are antagonists of calmodulin. The doses of 3×10^{-5} M trifluoperazine and 4.35×10^{-5} M W-7 were used since these doses produced a 50% inhibition of cellular cAMP production in our previous report (1). The cells were rinsed, and then incubated at 37 C for 10 min with forskolin (Nihon Kayaku Co., Tokyo, Japan) together with 5×10^{-4} M 3-isobutyl-1-methylxanthine (Nakarai Biochemicals, Tokyo, Japan) in 1 ml of the same media as that used during the preincubation. Control experiments were carried out in the same manner with the vehicle. After the incubation period, the cells were immediately immersed in 0.2 ml 0.1 N HCl to stop the reaction. The cells were collected according to our previous report (1). The supernatants were used to determine cAMP and the pellets were to measure protein contents.

Statistics

All values of cellular cAMP levels were compared by Scheffe's analysis of multiple variance and Student's t-test as appropriate. A P value less than 0.05 was considered significant.

RESULTS

Figure 1 shows the effect of preincubation with ouabain on cellular cAMP production in response to forskolin in rat renal papillary collecting tubule cells in culture. We have already demonstrated that forskolin causes a dose-dependent increase in cellular cAMP production (1). Exposure of cells to 1 x 10^{-5} M ouabain for 1 h significantly enhanced basal and 2 x 10^{-8} M forskolin-induced cellular cAMP levels. In contrast, only 10 min exposure to ouabain did not affect basal and forskolin-induced cellular levels of cAMP.

Figure 2 shows the inhibition of ouabain-enhanced cellular cAMP production in response to forskolin by a blocker of cellular Ca^{2+} uptake, cobalt (Co^{2+}). Forskolin increased cellular cAMP production in a dose-dependent manner. Such effect of forskolin was shifted to the left by the exposure to 1 x 10^{-5} M ouabain. The presence of 3 x 10^{-3} M Co^{2+}, however,

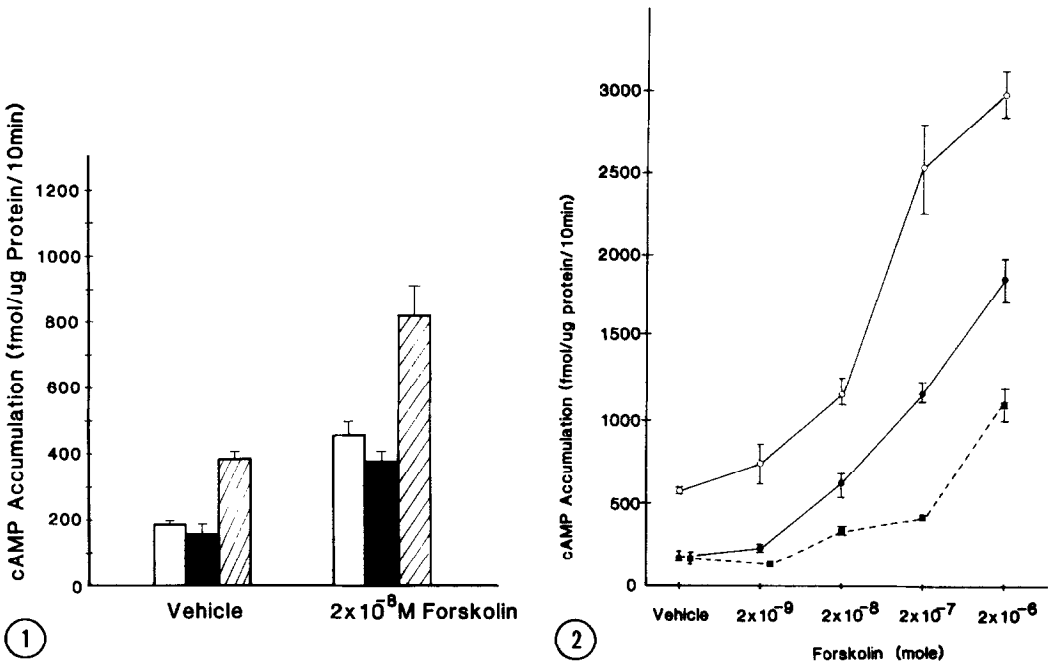

Figure 1 Effect of preincubation with ouabain on cellular cAMP production in response to forskolin in rat renal papillary collecting tubule cells in culture. Cells were untreated with ouabain (open bars), treated with 1 x 10^{-5} M ouabain during the 10 min experimental period (solid bars), or pretreated with 1 x 10^{-5} M ouabain for 1 h (hatched bars). Values are means ± SEM, n = 6.

Figure 2 Inhibition of ouabain enhancement of forskolin-induced cellular cAMP production by cobalt in rat renal papillary collecting tubule cells in culture. The cells were pretreated with control medium (●) or 1 x 10^{-5} M ouabain in the absence (○) or presence of 3 x 10^{-3} M Co^{2+} (■), and then incubated with forskolin for an additional 10 min. Values are means ± SEM, n = 6.

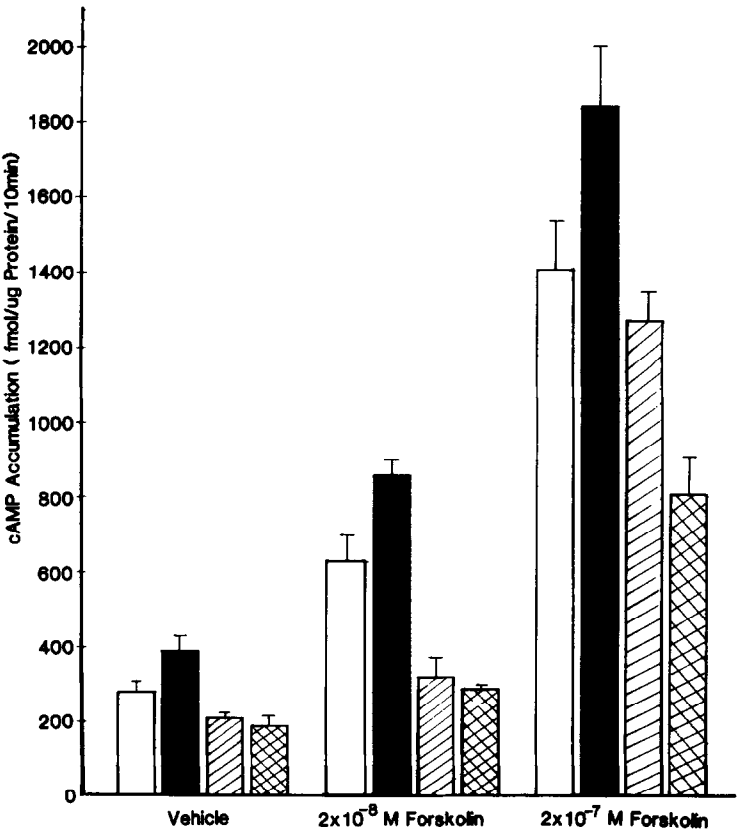

Figure 3 Effect of Ca^{2+}-free medium or verapamil on forskolin-induced cAMP production in the presence of ouabain in rat renal papillary collecting tubule cells in culture. Ca^{2+}-free medium contained 1 x 10^{-3} M EGTA. The cells were preincubated for 1 h with control medium (open bars), 1 x 10^{-5} M ouabain (solid bars), 1 x 10^{-5} M ouabain in Ca^{2+}-free medium (hatched bars), and a mixture of 1 x 10^{-5} M ouabain and 5 x 10^{-4} M verapamil (cross-hatched bars). Values are means ± SEM, n = 6.

totally blocked the effect of ouabain and forskolin on cellular cAMP production, since the response-curve shifted to the right.

Removal of extracellular Ca^{2+} or the presence of 5 x 10^{-4} M verapamil also attenuated the effect of ouabain on forskolin-stimulated cellular cAMP production (Fig. 3). Ouabain markedly augmented the cAMP response to forskolin. The ouabain's enhancement totally disappeared in cells exposed for 1 h to Ca^{2+}-free medium containing 1 x 10^{-3} M EGTA. The increment in cAMP production in response to forskolin was significantly less in cells exposed to 5 x 10^{-4} M verapamil than that in cells pretreated with Ca^{2+}-free medium. Verapamil blocked the effect of ouabain and forskolin on cellular cAMP production.

Figure 4 depicts the effect of calmodulin antagonists on ouabain-enhanced cAMP production in response to forskolin in rat renal papillary

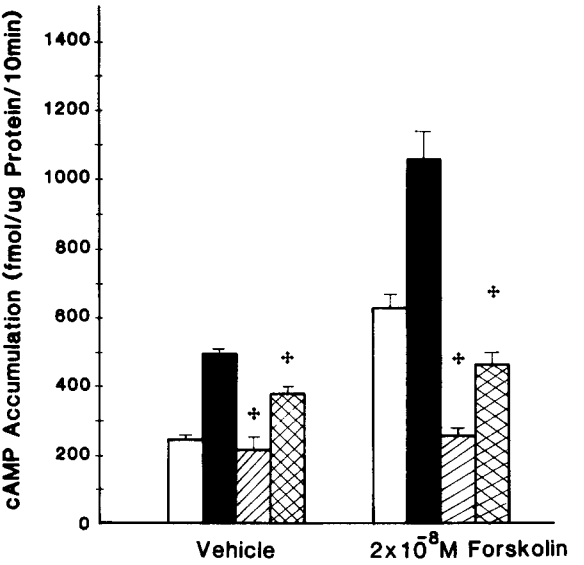

Figure 4 Effect of calmodulin antagonists on ouabain enhancement of forskolin-induced cAMP production in rat renal papillary collecting tubule cells in culture. The cells were preincubated for 1 h with control medium (open bars), 1 x 10^{-5} M ouabain (solid bars), a mixture of 1 x 10^{-5} M ouabain and 3 x 10^{-5} M trifluoperazine (hatched bars), or a mixture of 1 x 10^{-5} M ouabain and 4.35 x 10^{-5} M W-7 (cross-hatched bars). *P<0.01 vs. the ouabain group. Values are means ± SEM, n = 6.

collecting tubule cells in culture. The enhancement by 1 x 10^{-5} M ouabain of basal and 2 x 10^{-8} forskolin-induced cAMP production was blunted by pretreatment with either trifluoperazine or W-7. The inhibition by W-7 of cellular cAMP production was somewhat weaker than that by trifluoperazine when cells were exposed to 1 x 10^{-5} M ouabain.

DISCUSSION

The present study clearly demonstrated that ouabain enhanced basal and forskolin-induced cAMP production in rat renal papillary collecting tubule cells in culture. The enhancement by ouabain of cellular cAMP production in response to forskolin was blunted in cells pretreated with Co^{2+}, verapamil or Ca^{2+}-free medium containing 1 x 10^{-3} M EGTA. Ca^{2+}-free medium reduced the extracellular Ca^{2+} concentration to nominally zero mM, and verapamil and Co^{2+} are known as blockers of cellular Ca^{2+} uptake (12). As reported in our previous paper (8), ouabain increased Ca^{2+} influx, providing a 1.5-fold increase in $[Ca^{2+}]i$.

Ouabain blocks the Na^+, K^+-ATPase activity, increasing the intracellular Na^+ concentration. The Na^+/Ca^{2+} exchange system is reversible and can move Ca^{2+} in a direction opposite to that of the Na^+ electrochemical gradient (9). Therefore, the present effect of ouabain is suggested to depend on the extracellular Ca^{2+}, particularly on cellular Ca^{2+} uptake.

Calmodulin is an important factor in the regulation of forskolin-induced adenylate cyclase in renal papillary collecting tubule cells (1, 13). Two chemically dissimilar antagonists of calmodulin totally blunted the enhancement of cAMP production by ouabain. As mentioned by the previous study (8), ouabain increased $[Ca^{2+}]i$. Taken together, ouabain-enhanced production of cAMP is also regulated by calmodulin, which binds to the increased cellular free Ca^{2+} to form an active complex of Ca^{2+}-calmodulin.

Forskolin is a diterpene activator which activates adenylate cyclase at the catalytic unit. Our previous studies demonstrated that Ca^{2+} modulates the cellular action of AVP in renal papillary collecting tubule cells (1). The present study supports the conclusion that cellular free Ca^{2+} modulates the hormonal activation of adenylate cyclase activity in renal papillary collecting tubule.

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