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Angiotensin II Suppresses Water Absorption Through the Ventral Skin of Japanese Tree-Frogs *in vitro*.

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ABSTRACT—We previously described two different water absorption systems in the ventral skin of the Japanese tree-frog, *Hyla arborea japonica*: i.e., a rapid enhanced flow, which is observed in dehydrated tree-frogs or those stimulated by adrenaline β-agonists or vasotocin, and a slow basal flow, which is observed in normally hydrated frogs during the non-breeding season. The rapid flow is completely blocked by ouabain, which has no effects on the slow basal flow. In the present experiment, we show that the vaso-constrictive hormone angiotensin II completely inhibits basal water absorption, but has no effect on rapid water absorption. These results confirm our previous finding that the two water absorption systems in the ventral skin of the Japanese tree-frog are independent of each other.

INTRODUCTION

Hydromineral regulation in amphibians has been investigated in the urinary bladder and skin as it relates to osmotic integrity in these animals, which live in diverse environments [1, 2, 10, 23]. Most terrestrial anurans intake water mainly through a portion of the ventral skin called the pelvic patch. Water absorption through the ventral skin in some anurans can be enhanced by keeping the animals under dehydrating conditions, or by stimulating their ventral skin with sympathomimetic agents or antidiuretic hormones (ADH) [7, 15, 17, 21]. Ventral skin isolated from Japanese tree-frogs kept under normally hydrated conditions absorbs water at a slow but constant rate of 30 μl/cm^2/min (basal flow) during the non-breeding season. However, during the breeding season, from April to October, there is no substantial water absorption through the skin [19]. Water absorption through the excised skin of hydrated tree-frogs in the non-breeding season can be greatly enhanced by stimulation with a β-adrenaline agonist, isoproterenol (ISO) or vasotocin. Such treatment can increase water absorption about five times higher than basal water absorption in normally hydrated tree-frogs [19]. This rapid water absorption stimulated by β-agonists or ADH is completely blocked by ouabain, a specific Na^+, K^+-ATPase inhibitor, which does not affect basal water absorption through non-stimulated skin [19]. The β-antagonist propranolol, which naturally blocks the water absorption enhanced by ISO stimulation, also has no effects on basal water absorption through non-stimulated skin in normally hydrated frogs [19]. These previous findings suggest that the ventral skin of Japanese tree-frogs contains two different water absorption systems. However, there have been no reports of possible mediators of basal water absorption except prolactin, which produces non-specific suppression of water absorption through tree-frog skin [19].

Several reports have indicated that angiotensin II, a hormone that controls Na^+ and water reabsorption in the kidney of tetrapod vertebrates and in the cutaneous water absorption of anurans, may be involved in these water absorption systems [3, 5, 11, 20]. In all of these reports, angiotensin II has been considered to enhance cutaneous water permeation. Angiotensin II is also known to promote water drinking in tetrapods, except amphibians [16]. Amphibians usually do not take water orally, and drinking behavior is not induced by angiotensin II [13]. In the present study, we examined the effect of [Val^5]-angiotensin II on water absorption through the skin of Japanese tree-frogs *in vitro* and found that this agent completely inhibited basal water absorption, but only slightly inhibited stimulated water absorption.

MATERIALS AND METHODS

Japanese tree-frogs (*Hyla arborea japonica*) were collected from the field in summer and kept under laboratory conditions until use. All of the tree-frogs used in this experiment were kept under a 12 hr/12 hr light/dark cycle at 25°C, and were allowed to feed on mealworms with access to water ad libitum. Experiments were carried out in the non-breeding season of the Japanese tree-frog (November to March), since the skin of this frog becomes impermeable to water outside of this period [19]. The animals were double-pithed and decapitated before the skin was removed. Isolated skin that was treated with adrenoceptor agonist or ADH was referred to as stimulated skin, whereas skin that was treated with bathing solution alone was used as control skin. The bathing solution for the serosal side of the skin consisted of NaCl : 110 mM, KCl : 1.1 mM, $CaCl_2$: 1.1 mM, $MgCl_2$: 1.0 mM, and glucose : 2.5 mM, and was adjusted pH to 7.4 with $NaHCO_3$: 2.4 mM, Na_2HPO_4 : 0.07 mM, to give an osmolarity of 230 mOsM/l. The mucosal side of the skin was bathed in deionized and distilled water. Thus, the osmotic difference between

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the serosal and mucosal media was about 230 mOsM/l. The isolated skin was mounted on a device modified from a Millipore plastic filter holder (Swinex, Millipore Corp.), and water movement from the mucosal side to the serosal side of the skin was directly read from the meniscus of a microliter pipette (Pyrex) attached to the serosal chamber of the holder, as has previously been described [19]. [Val^5]-angiotensin II (Peptide Institute Inc., Japan), arg-vasotocin (Sigma), isoproterenol (Sigma) and ouabain (Sigma) were administered to the serosal solution. In all experiments, measurements were started 30 min after the skin was mounted on the equipment to ensure the agent or agents had sufficient time to penetrate the skin. Each experiment was repeated five times and the significance of differences was evaluated by Student's *t*-test.

RESULTS

Excised ventral skin from Japanese tree-frogs kept under hydrated conditions usually showed basal water absorption of 15 μl/cm^2/hr during the non-breeding season from November to March (Fig. 1). Water absorption through the skin was also very sensitive to the antidiuretic hormone arginine-vasotocin (AVT) and the sympathomimetic agent isoproterenol (ISO). Water absorption with these agents ranged from 80 to 90 μl/cm^2/hr, which was five to six times greater than the basal absorption with non-stimulated control skin (Fig. 1). The addition of both of these mediators simultaneously caused no further enhancement of water absorption. The

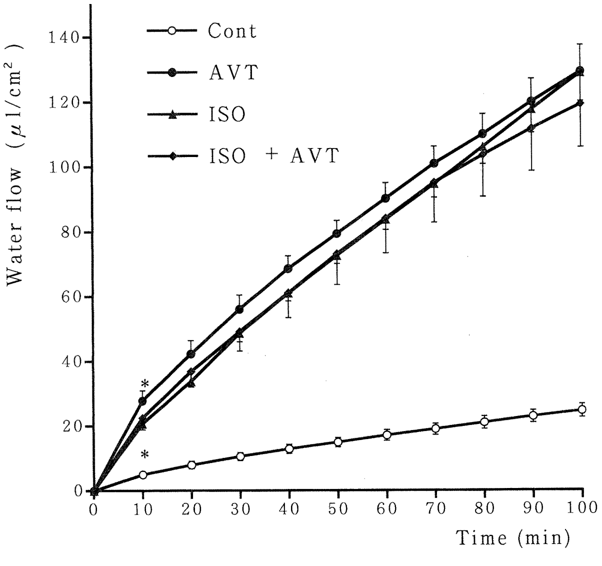

FIG. 1. Effects of isoproterenol (ISO) and arginine-vasotocin (AVT) on water absorption through the excised ventral skin of the Japanese tree-frog. These agents were administered by dissolving them in the serosal solution. Both of the agents strongly enhanced water absorption, compared to that in non-stimulated skin (Cont), while the combined administration of ISO and AVT gave no additional enhancement. No significant differences were observed among stimulated absorptions, all of which were significantly higher than the basal level ($P<0.001$). All agents were used at 10^{-6} M. Vertical bars indicate the range of the standard error. Asterisk(*) indicates a point after which each value was significantly higher ($P<0.001$) against the control value.

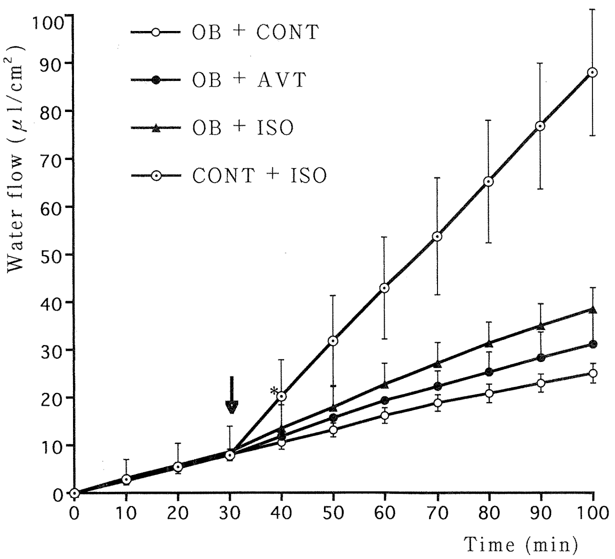

FIG. 2. Effects of ouabain (OB, 10^{-6} M) on enhanced water absorption due to ISO or AVT, and in control skin (Cont). Ouabain had no effect on basal water absorption in non-stimulated control skin. No significant increase was observed by the addition of stimulants to ouabain-treated skin. The arrow indicates the point where ISO was added to the control skin during the experiment. ISO-stimulated absorption was significantly suppressed by ouabain ($P<0.001$) (○ and ▲). With ouabain treatment, the stimulated water flow was not significantly greater than the basal flow. For clarity, some of overlapping lines and bars were omitted from the graph. Asterisk(*) indicates a point after which each value was significantly higher ($P<0.001$) against the control value.

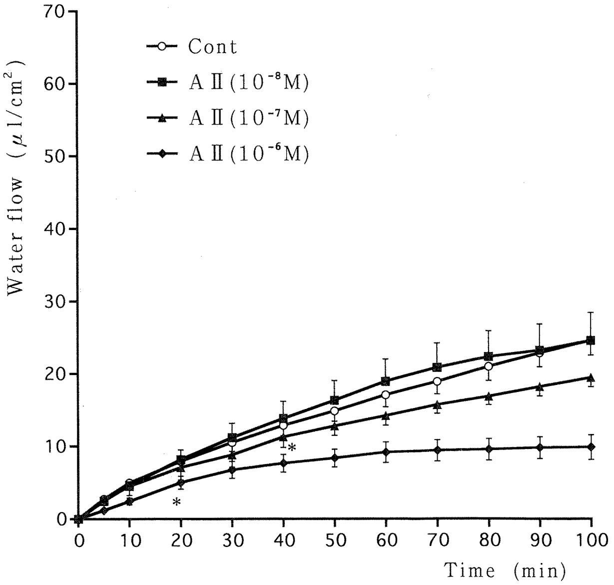

FIG. 3. Time course of the effects of angiotensin II (A II) on basal water absorption. A II was added to the serosal solution at time 0. Water absorption was suppressed dose-dependently by A II ($P<0.01$ between each hormone-treated preparation). At a concentration of 10^{-6} M, A II completely inhibited water absorption within 60 min after administration. In contrast, 10^{-8} M A II had no significant effect on water flow versus the control. Cont: control skin without hormone administration. Asterisk(*) indicates a point after which each value was significantly lower ($P<0.001$) against the control value.

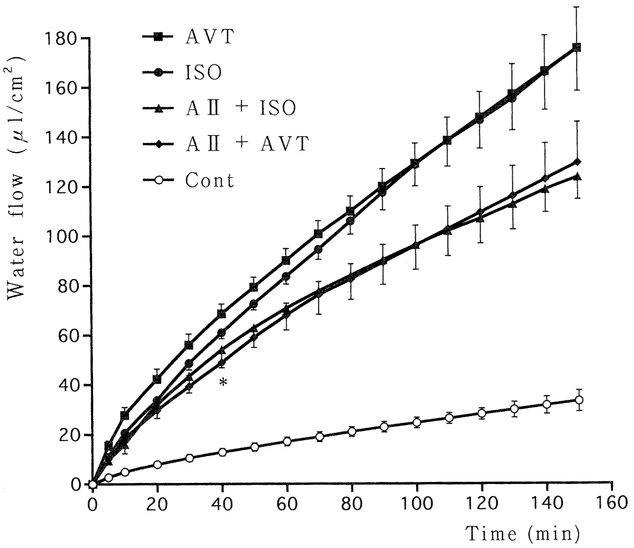

FIG. 4. Effects of angiotensin II at 10^{-6} M (A II) on the enhancement of water absorption by ISO or AVT. A II significantly suppressed the enhancement of water absorption by either ISO or AVT ($P<0.001$). However, this suppression was considered to be caused by complete cessation of the contribution of the basal water absorption to the system. Asterisk (*) indicates a point after which each value of A II treated specimens was significantly lower ($P<0.001$) than the respective stimulated specimen.

effects of AVT and ISO were completely inhibited by the presence of 10^{-6} M ouabain in the serosal bathing solution. However, ouabain had no effect on non-stimulated basal absorption (Fig. 2).

On the other hand, administration of angiotensin II (A II) suppressed basal water absorption dose-dependently in the control skin, and inhibited it completely at a concentration of 10^{-6} M after 60 min of treatment (Fig. 3). The enhancement of water absorption by AVT or ISO was also reduced to some extent by the administration of A II (Fig. 4). However, this inhibition was only partial and even a high dose of A II (such as 10^{-6} M) did not completely prevent water absorption through the skin. The maximal reduction in the enhanced water absorption due to A II was equivalent to the level of the basal water absorption,(Figs. 3 and 4). At 10^{-8} M or less, A II had no significant effects on either basal or enhanced water absorption.

DISCUSSION

Cutaneous water absorption in terrestrial anurans has been shown to be stimulated by antidiuretic hormones [4, 7] or β-adrenoceptor agonists. These mediators of water absorption are especially conspicuous in toads [8, 11, 21, 22] and Hylidae frogs [19, 23]. We previously reported that the enhancement of water absorption by β-agonist or ADH in Japanese tree-frogs is completely inhibited by ouabain, a specific Na^+, K^+-ATPase inhibitor, suggesting that this water absorption is induced by activation of the sodium pump on the basolateral membrane of the outermost cells in the epidermis [19]. However, basal water absorption in non-stimulated skin from tree-frogs kept under normally hydrated conditions seems to be unaffected by ouabain, which suggests that the sodium pump is not involved in the basal water absorption commonly observed in the control skin of normally hydrated Japanese tree-frogs during the non-breeding season. We found no evidence that the alpha receptor was involved in cutaneous water absorption in Japanese tree-frogs (not shown), although an α_2-adrenoceptor agonist has been reported to inhibit water permeation in toad skin [8].

In the present experiment, angiotensin II, a hydromineral mediator which promotes sodium and water retention in kidney tubules, completely inhibited basal water absorption in normally hydrated control frogs. Angiotensin II is also known to induce water drinking behavior in tetrapods [16]. Although this hormone causes no such drinking behavior in amphibians [12], several reports have suggested that it enhances water absorption through the skin in toads [3, 5, 10, 13, 20]. The present finding that angiotensin II, at a concentration of 10^{-6} M, completely inhibited basal water absorption in the skin of Japanese tree-frogs kept under normally hydrated condition appears to conflict with previous results. This hormone seems to have no significant effects on the enhanced water absorption due to neuronal or hormonal mediators. The apparent suppression of enhanced absorption in this experiment by angiotensin II (Fig. 4) might be a result of the complete inhibition of the basal absorption which naturally contributes to the enhanced absorption.

Both of the stimulants that were used to enhance water absorption, i.e., β-agonist and ADH, have been shown to promote cAMP production, which may lead to activation of the sodium pump [6, 14, 18]. On the other hand, basal water absorption in the Japanese tree-frog is not affected by inhibition of the sodium pump, as shown here and in previous reports [19]. This suggests that basal water absorption in Japanese tree-frogs may not be regulated by a system that involves cyclic AMP production and activation of the sodium pump. The β-adrenoceptor antagonist propranolol also did not affect basal water absorption in the skin from normal hydrated tree-frogs[19]. We recently found that the inhibitory effect of angiotensin II on basal absorption is mimicked by a phorbol ester that stimulates protein kinase C (unpublished observations). All of these findings indicate that the Japanese tree-frog has two different water absorption systems, each of which is activated by a different intracellular effector pathway: i.e., basal absorption inhibited by angiotensin II which may activate protein kinase C, and enhanced absorption, which is induced by β-adrenoceptor agonist or by ADH via Na^+, K^+-ATPase activation.

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