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Correlation between aquaporin and water permeability in response to vasotocin, hydrin and β-adrenergic effectors in the ventral pelvic skin of the tree frog *Hyla japonica*

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SUMMARY

The ventral pelvic skin of the tree frog *Hyla japonica* expresses two kinds of arginine vasotocin (AVT)-stimulated aquaporins (AQP-h2 and AQP-h3), which affect the capacity of the frog's skin to absorb water. As such, it can be used as a model system for analyzing the molecular mechanisms of water permeability. We investigated AQP dynamics and water permeability in the pelvic skin of *H. japonica* following challenge with AVT, hydrins (intermediate peptides of pro-AVT) and β-adrenergic effectors. In the *in vivo* experiment, both AQP-h2 and AQP-h3 proteins were translocated to the apical plasma membrane in the principal cells of the first-reacting cell (FRC) layer in the pelvic skin following challenge with AVT, hydrin 1 and hydrin 2, thereby increasing the water permeability of the pelvic skin. The β-adrenergic receptor agonist isoproterenol (IP) and its anatagonist propranolol (PP) in combination with AVT or hydrins were used as challenge in the *in vitro* experiment. IP increased water permeability whereas PP inhibited it, and both events were well correlated with the translocation of the AQPs to the apical membrane. In the PP+AVT-treated skins, labels for AQP-h2 and AQP-h3 were differentially visible among the principal cells; the apical plasma membrane of some cells was labeled while others were not, indicating that the response of PP or AVT is different from cell to cell. These results provide morphological evidence that the principal cells of the FRC layers may have two kinds of receptors: a V2 receptor and β-adrenergic receptor.

Key words: aquaporin, hydrosmotic response, vasotocin, hydrin, β-adrenergic effectors, ventral pelvic skin, immunohistochemistry, the tree frog, *Hyla japonica*.

INTRODUCTION

Anuran amphibians are adapted to living in a diverse range of water environments. A general classification of anurans according to habitat distinguishes four groups, i.e. aquatic, semi-aquatic, terrestrial and arboreal species (Hillman et al., 2009). Anurans have developed a number of unique physiological systems for maintaining water balance in these diverse habitats (Bentley and Main, 1972; Bentley and Yorio, 1979). For example, most adult anuran amphibians do not drink water through their mouth. This is particularly true for many terrestrial and arboreal species, such as *Hyla* (arboreal) and *Bufo* (terrestrial); these species utilize a region in the posterior or pelvic region of the ventral skin that is specialized for rapid rehydration from shallow water sources or moist substrates. In addition, periods of terrestrial activity can be prolonged by the reabsorption of dilute urine from the bladder. The degree to which these adaptations have been developed varies among the different anurans. For example, the terrestrial-environment-adapted species have high water permeability in their ventral pelvic skin and a high capacity for urine storage in their urinary bladder whereas the pelvic skin of an aquatic species, such as *Xenopus laevis*, has a low water permeability (Bentley and Main, 1972). In addition, the stimulation of water permeability of the pelvic skin by arginine vasotocin (AVT) is higher in arboreal and terrestrial species than in aquatic species (Yorio and Bentley, 1978).

Brown et al. measured a correlation between the aggregation of intramembrane particles (termed aggrephore) and water flow in toads skin in response to the mammalian antidiuretic hormone arginine vasopressin (Brown et al., 1983). These particles were subsequently shown to be a class of integral membranes [termed aquaporins (AQPs)] that form selective water channels in the plasma membranes in various cells of various organisms (Preston et al., 1992).

In mammals, 13 isoforms of AQPs (AQP0–AQP12) have been identified to date and have been characterized by cloning and sequencing their cDNA (Ishibashi et al., 2000; Takata et al., 2004). A subfamily of AQPs (termed aquaglyceroporins) that form membrane pores, which are permeated by glycerol and urea, has also been reported (Ishibashi et al., 2000; Agre, 2006). Our group recently discovered three forms of AVT-stimulated AQP in the tree frog *Hyla japonica*: AQP-h2 in the ventral pelvic skin and urinary bladder (Hasegawa et al., 2003), AQP-h3 in the pelvic skin (Tanii et al., 2002) and AQP-h2K in the kidney (Ogushi et al., 2007). The expression of AQP-h2 and AQP-h3 in the pelvic skins of tree frogs raises the interesting question of whether other factors that stimulate water permeability and absorption by anurans involve a similar insertion of AQPs in the apical membrane.

Hydrin is an intermediate peptide derived from a pro-vasotocin-neurophysin precursor. It plays a pivotal role in the water absorption/reabsorption process in the skin and bladder but is devoid of antidiuretic activity in the kidney (Michel et al., 1993; Rouille et al., 1989). Hydrin 2 (vasotocinyl-Gly) is present in an amount equal to that of AVT in the neurohypophysis of Ranidae and Bufonidae. By contrast, the aquatic frog *Xenopus laevis* secretes

hydrin 1 (vasotocinyl-Gly-Lys-Arg) as well as AVT. Both peptides are generated from a downregulation in the processing, i.e. hydrin 1 by a decrease in carboxypeptidase E activity, and hydrin 2 by a reduction in the activity of the α-amidating enzymatic system. These hydrins are found in anuran amphibians, but not in other vertebrates, raising the question of whether hydrin has a stimulatory potency in the translocation of frog AQPs to the apical membrane.

Water permeability in the pelvic skin of frogs is considered to be regulated by at least two systems: the first is *via* the AVT V2 receptor, and the other is *via* the β-adrenergic receptor (Brown et al., 1980; De Sousa and Grosso, 1982). Our group has shown that both AQP-h2 and AQP-h3 proteins are translocated in response to AVT to the apical plasma membrane of principal cells in the outermost granular layer [i.e. the first-reacting cell (FRC) layer] of the pelvic skin in tree frogs (Hasegawa et al., 2003).

In the study reported here, we examined the effect of AVT, hydrin and effectors of the β-adrenergic receptor on the water permeability and translocation of both AQP-h2 and AQP-h3 in the FRC layer of two pelvic skins of the tree frog.

MATERIALS AND METHODS

Animals

Adult male tree frogs (*Hyla japonica* Günter) were captured in a field near our university (Shizuoka University, Shizuoka, Japan) and kept under laboratory conditions where they were fed crickets. On average, the frogs used in this study had a body length of approximately 2.9 cm and weighed approximately 2.6 g. The ventral pelvic skins were removed under anesthesia (MS 222; Nacalai tesque, Kyoto, Japan) and then processed for experiments involving hormone or reagent treatments. All animal experiments were carried out in compliance with the Guide for Care and Use of Laboratory Animals of Shizuoka University.

Experimental protocol for treatment with chemical reagents

For the *in vivo* experiments, the frogs were bathed to above two-thirds of their ventral skin in distilled water for 30 min and then injected with 10 µl 0.65% (w/v) NaCl saline containing 10^{-6} mol l^{-1} [Arg^8]-vasotocin (Peptide Institute, Inc., Osaka, Japan), –hydrin 1 or –hydrin 2 (Bachem, Bubendorf, Switzerland) per 1 g of body mass. Following the injection, the body mass of the frogs was measured chronologically on an analytical balance (Sartorius, Tokyo, Japan).

For the *in vitro* experiments, the frogs were rinsed in water for 30 min before being killed as described above and the pelvic skins removed. The apparatus for measuring water flow consists of two chambers with a 1 cm-diameter hole at the center of the wall separating the two chambers, which was a modification of the device by Kohno et al. (Kohno et al., 2004). The isolated skin was washed with Ringer solution and then mounted over the hole. The chamber on the serosal side of the skin was filled with Ringer solution: 11.301 mmol l^{-1} NaCl, 1.877 mmol l^{-1} KCl, 1.081 mmol l^{-1} $CaCl_2$, 0.064 mmol l^{-1} NaH_2PO_4 and 1.429 mmol l^{-1} $NaHCO_3$ having an osmolarity of 220 mosmol, while that on the mucosal side of the skin was filled with water. Water movement from the mucosal side to the serosal side of the skin was measured directly using a 0.1 ml pipette horizontally attached the serosal chamber of the apparatus. The isolated pelvic skins were preincubated for 30 min in Ringer solution as described above or in a Ringer solution containing 10^{-8} mol l^{-1} AVT, hydrin 1 or hydrin 2. Following the addition of each of the solutions to the serosal side of the chamber, water movement was measured using the pipette. Applying the same procedure, we added Ringer solution containing 10^{-8} mol l^{-1} of the β-adrenergic receptor agonist isoproterenol (IP; Sigma-Aldrich, Tokyo, Japan) and its antagonist propranolol (PP; Sigma-Aldrich), respectively, to the serosal side of the chamber to examine the effects of these substances on the actions of AVT, hydrin 1 and hydrin 2. After incubation, the skin specimens were examined by immunofluorescence microscopy.

Immunofluorescence

The pelvic skins used in the *in vivo* and *in vitro* experiments were quickly fixed overnight in periodate-lysine-paraformaldehyde (PLP) fixative, dehydrated and embedded in Paraplast. Four-µm sections were cut and mounted on gelatin-coated slides. The deparaffinized sections were rinsed with phosphate-buffered saline (PBS). Double-immunofluorescence labeling was carried out according to Hasegawa et al. (Hasegawa et al., 2003). In brief, for the double-immunofluorescence labeling of AQP-h2 and AQP-h3, sections were incubated with a mixture of guinea pig anti-AQP-h2 [ST-140; 1:5,000 (Hasegawa et al., 2003)] and rabbit anti-AQP-h3 [ST-141; 1:10,000 (Tanii et al., 2002)], and then reacted with a mixture of indocarbocyanine (Cy3)-labeled donkey anti-guinea pig IgG (1:400; Jackson Immunoresearch, West Grove, PA, USA), Alexa 488-labeled donkey anti-rabbit IgG (1:200; Molecular probes, Eugene, OR, USA) and DAPI. Rabbit anti-*Hyla* AQP-h3BL serum (ST-184) was also used for labeling the basolateral membrane of the principal cells (Akabane et al., 2007). Specimens were examined with an Olympus BX61 microscope equipped with a BX-epifluorescence attachment (Olympus Optical Co., Tokyo, Japan).

Statistical analysis

Statistical analysis was performed by Duncan's multiple range test.

RESULTS

In vivo experiment

The body mass of the frogs increased significantly with time following the injection of AVT, reaching a body mass approximately 1.15-fold greater than that of the control frogs after 60 min (P<0.01) (Fig. 1). Hydrin 1 and hydrin 2 were equally as effective as AVT in stimulating the increase in body mass (Fig. 1). By contrast, the control frogs injected with 0.65% NaCl solution did not show any change in body mass within the experimental interval. In the control group, the labels for AQP-h2 and AQP-h3 were observed in the

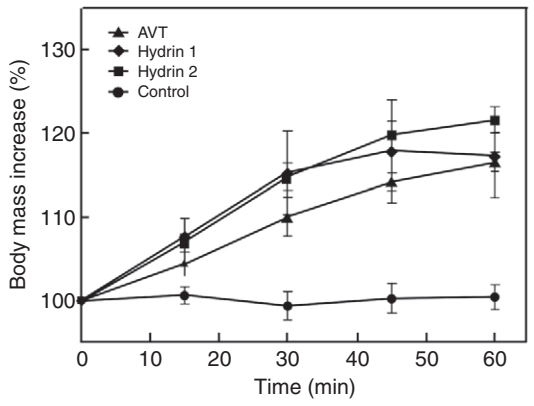

Fig. 1. Time course of water permeability across the ventral pelvic skin *in vivo* following challenge with arginine vasotocin (AVT), hydrin 1 and hydrin 2. The body mass of these frogs challenged with each these hormones increases significantly with time. At 60 min there are significant differences between the control group and the hormone-treated groups (P<0.01) but not among the hormone-treated groups.

basolateral membrane of the principal cells in the FRC layer (Fig. 2A–C). In comparison, when sections from the AVT-injected frogs were labeled with anti-AQP-h2 or anti-AQP-h3, these labels were visible in the apical membrane of the principal cells in the FRC layer and throughout the entire plasma membrane of the principal cells underlying the FRC layer (Fig. 2D–F). Similar labeling patterns for AQP-h2 and AQP-h3 were observed on sections from the hydrin 1- and hydrin 2-injected frogs (Fig. 2G–L).

In vitro experiment

The effect of AVT was to gradually stimulate the water permeability of the isolated pelvic skins with increasing incubation time; by contrast, the water permeability of the control pelvic skins did not increase (Fig. 3). Hydrin 1 and hydrin 2 also were effective in increasing the water permeability of the isolated pelvic skins but at slightly lower levels than those observed for the AVT-treated skins (Fig. 3). At the end of the experimental period (30 min), the water

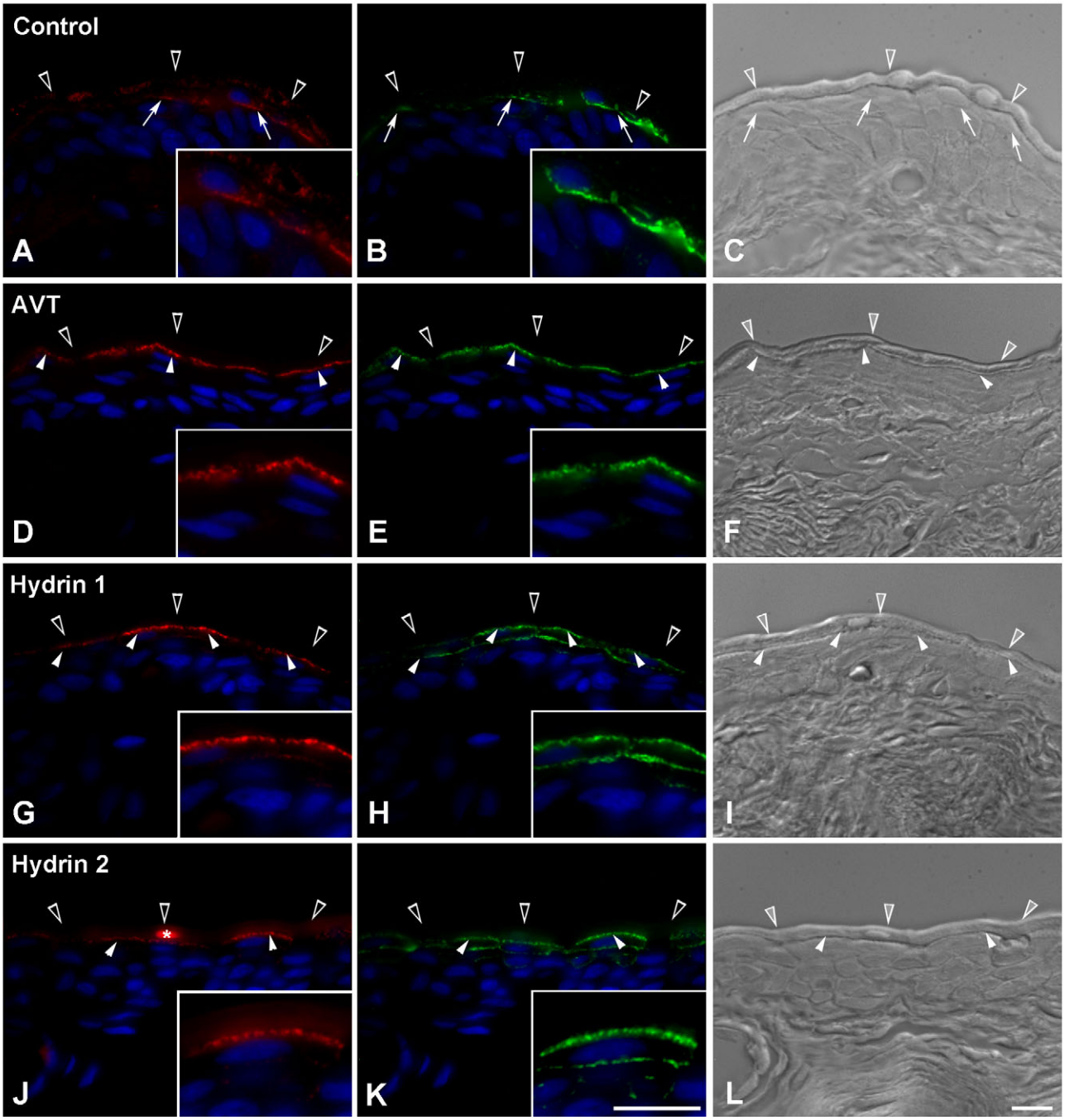

Fig. 2. Fluorescence images of the pelvic skin in the *in vivo* experiment. The labels for AQP-h2 (red) and AQP-h3 (green) are observed in the basolateral plasma membrane in the principal cells in the control group (A–C). In the arginine vasotocin (AVT) (D–F)-, hydrin 1 (G–I)- and hydrin 2 (J–L)-treated groups, labels for AQP-h2 (red) and AQP-h3 (green) are visible in the apical plasma membrane in the principal cells of the first-reacting cell (FRC) layers of the pelvic skin. C, F, I and L are the corresponding Nomarski differential interference images. Insets in A, B, D, E, G, H, J and K show higher magnification of each of the figures. Arrows and white arrowheads indicate the basolateral and apical plasma membrane, respectively. Asterisk: non-specific label. White open arrowheads: cornified layer. Nucleus: blue color. Scale bars, 10 μm.

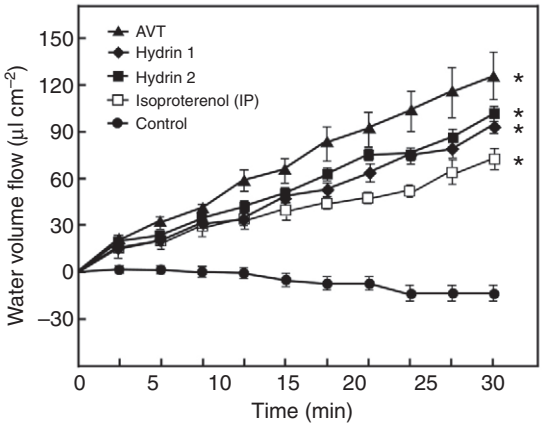

Fig. 3. Time course of water permeability across the isolated ventral pelvic skin *in vitro* following challenge with arginine vasotocin (AVT), hydrin 1, hydrin 2 and isoproterenol (IP). The amount of water in the chamber on the serosal side increased significantly with time following the addition of the hormones. **P*<0.01 *vs* control.

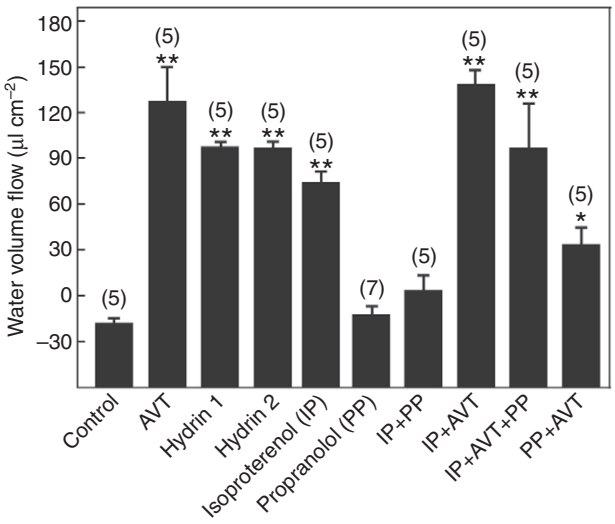

Fig. 4. Comparison of water permeability in the *in vitro* experiment at the end of the incubation period (30 min) among the arginine vasotocin (AVT)-, hydrin 1-, hydrin 2-, isoproterenol (IP)-, propranolol (PP)-, IP+PP-, IP+AVT-, IP+AVT+PP- and PP+AVT-treated pelvic skins. AVT, hydrin 1, hydrin 2 and IP significantly increased water permeability when compared with the control (*P*<0.01). By contrast, there was no significant difference among the PP- and IP+PP-treated skins and the control skins. The IP+AVT and the IP+AVT+PP treatments resulted in a strong increase of water permeability, similar to the AVT-treatment. However, the PP+AVT treatment significantly decreased water permeability when compared with the AVT group but water permeability was high than that of the control skins. The number of individuals used in each group is indicated in parentheses. **P*<0.05 or ***P*<0.01 *vs* control.

permeability of the AVT-, hydrin 1- and hydrin 2-treated skins had increased significantly compared with the control group (*P*<0.01) (Fig. 4). However, there was no significant difference among the AVT-, hydrin 1- and hydrin 2-treated groups. In each of the histological sections from the control group, labels for AQP-h2 and AQP-h3 were observed near or in the basolateral membrane in the FRC layer whereas the label for AQP-h3BL, which is expressed constitutively in the basolateral plasma membrane (Akabane et al., 2007), was visible at the same sites (Fig. 5A–C). In the AVT-, hydrin 1- and hydrin 2-treated groups, however, labels for AQP-h2 and AQP-h3 were intensely visible in the apical plasma membrane of the principal cells in the FRC layer (Fig. 5D,E,G,H,J,K). In the AVT-treated group, many spot-like AQP-h3-positive reactions were found in the cytoplasm of the principal cells, and the same intense positive reaction as described above was also visible throughout the entire plasma membrane of the principal cells underlying the FRC layer (Fig. 5D–F).

Water permeability in the IP-treated pelvic skin specimen increased in a similar manner to that observed for the hydrin-treated skin. At the 30-min incubation point, water permeability of the IP-treated pelvic skin was significantly lower than that of the IP+AVT-treated skin (*P*<0.01) (Fig. 4). In the PP-treated group, the water permeability was inhibited up to the control levels (Fig. 4). In turn, when the pelvic skins were challenged with IP+PP, water permeability was not as low as that of the PP-treated and control skins (Fig. 4). The water permeability of the IP-treated pelvic skins was lower than that of the AVT-treated skins (*P*<0.01) but there was no significant difference in the water permeability between the AVT- and the IP+AVT-treated skins in terms of water permeability (Fig. 4). The treatment with PP+AVT induced a mean increase in water permeability *via* the isolated pelvic skins but the levels were higher than those observed in the control group (Fig. 4).

Similar stimulatory and inhibitory experiments with IP or PP using hydrin 1 and hydrin 2 produced the same results as those using AVT (data not shown).

Intense labels were observed in the apical membrane of the principal cells in the FRC layers when sections of the IP-treated pelvic skins were labeled with anti-AQP-h2 or anti-AQP-h3. Similar labeling was only found in the AVT-treated skins (Fig. 5D–F, Fig. 6A–C).

Increased water permeability was not observed in the PP-treated and control skins, which is consistent with the distribution of AQP-h2 and AQP-h3 labels, which were visible in the basolateral plasma membrane (Fig. 6D–F). In the PP-treated group, labels for AQP-h2 and AQP-h3 were visible in the basolateral plasma membrane of the principal cells in the FRC layer (Fig. 6D–F).

When the sections of the IP+AVT-treated skins were labeled for AQP-h2, more intense labels were found in the apical membrane of the principal cells. Similar results were obtained in the IP+AVT+PP-treated pelvic skins (data not shown).

When the sections of the PP+AVT-treated group were labeled for AQP-h2 and AQP-h3, positive labels were found in the apical plasma membrane of several principal cells but not in all of the cells in the FRC layer (Fig. 5D–F, Fig. 6M–O). When all of the reagents were used, labels for AQP-h3BL were visible in the basolateral plasma membrane in the principal cells (Fig. 5C,F,I,L and Fig. 6C,F,I,L,O).

DISCUSSION

The present study demonstrated that hydrins as well as AVT stimulate water permeability across the ventral pelvic skin, *in vivo* and *in vitro*, by translocation of AQP-h2 and AQP-h3 from the cytoplasmic pool to the apical plasma membrane of principal cells in the FRC later. This process of water permeability is induced by two AQPs (AQP-h2 and AQP-h3), which translocate from the cytoplasmic pool to the apical plasma membrane of the principal cells in the FRC layer (Tanii et al., 2002; Hasegawa et al., 2003). AVT binds to V2 receptors and stimulates the formation of cyclic AMP and protein kinase A, thereby moving from the cytoplasmic pool to the apical plasma membrane through the phosphorylation

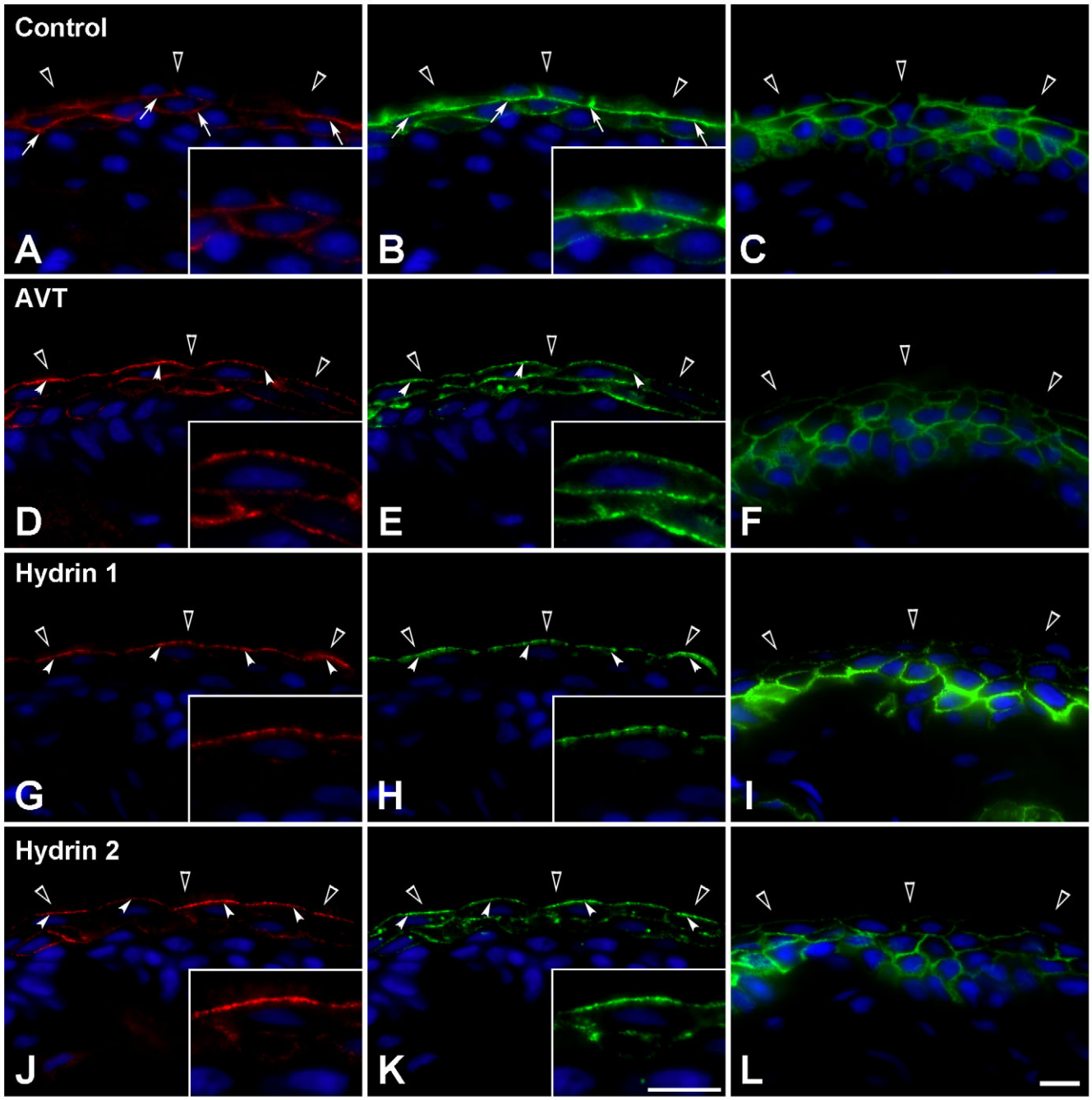

Fig. 5. Fluorescence images of the pelvic skin in the *in vitro* experiment. In the control skins, the labels for AQP-h2 (red) (A) and AQP-h3 (green) (B) are observed in the basolateral plasma membrane in the principal cells. In the arginine vasotocin (AVT)-, hydrin 1- and hydrin 2-treated skins, labels for AQP-h2 (D, G and J) and AQP-h3 (E, H and K) are visible in the apical plasma membrane (white arrows) in the principal cells in the first-reacting cell (FRC) layers of the pelvic skin. AQP-h3BL is constitutively expressed in the basolateral membrane in all the groups (C, F, I and L). Insets in A, B, D, E, G, H, J and K show higher magnification of each of the figures. White open arrowheads: cornified layer. Nucleus: blue color. Scale bars, 10 μm.

of AQP-h2 and/or AQP-h3 protein (Hasegawa et al., 2005). Concerning hydrin, Acher et al. (Acher et al., 1997) propose that the primary effect of AVT at its physiological concentration is antidiuresis whereas hydrins have a higher affinity for V2 receptors in the skin and bladder in order to stimulate rehydration and the utilization of bladder water. Our results provide experimental evidence supporting part of this hypothesis by showing that hydrins have a stimulatory effect on water permeability across the pelvic skin. This is the first report of a study showing a good correlation between hydrin and AQP protein in the ventral pelvic skin of the tree frog. However, further investigation is necessary to elucidate whether hydrins affect the translocation of AVT-stimulated AQP, AQP-h2K in the kidney (Ogushi et al., 2007). We also found that IP, a β-receptor agonist, and AVT have equal effects on water permeability, *in vitro*. This finding is consistent with *in vivo* studies on the hydrated toads, *Bufo cognatus*, dehydrated toads, *Bufo bufo*,

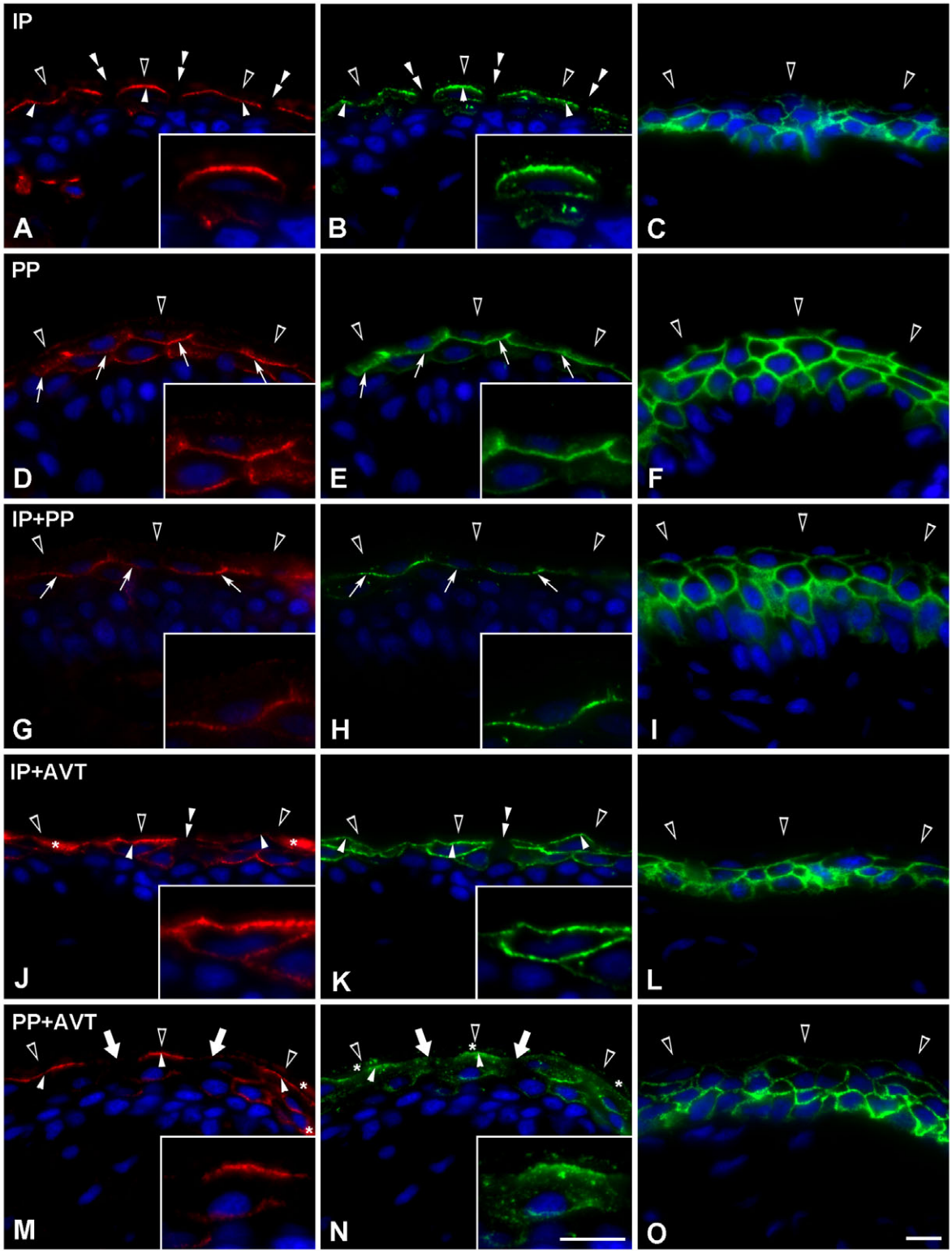

Fig. 6. Fluorescence images of the pelvic skin in the *in vitro* experiment. In the isoproterenol (IP)-treated skins, labels for AQP-h2 (red) (A) and AQP-h3 (green) (B) are visible in the apical plasma membrane. In the propranolol (PP)- and IP+PP-treated skins, the labels for AQP-h2 (D and G) and AQP-h3 (E and H) are observed in the basolateral plasma membrane in the principal cells. In the IP+arginine vasotocin (AVT)-treated skins, labels for AQP-h2 (J) and AQP-h3 (K) are visible in the apical plasma membrane in the principal cells in the first-reacting cell (FRC) layers of the pelvic skin. In the PP+AVT-treated skins, labels for AQP-h2 (M) and AQP-h3 (N) are observed differentially among the principal cells; one label is in the apical plasma membrane (white arrowheads) and the other is not in the apical membrane (large arrows) in the FRC layer. AQP-h3BL is localized in the basolateral membrane in all of the groups (C, F, I, L and Q). Arrows and white arrowheads indicate the basolateral and apical plasma membrane, respectively. Double arrowheads and asterisks indicate mitochondria-rich cells and non-specific label in the cornified cell layer or red blood cells, respectively. Insets in A, B, D, E, G, H, J, K, M and N show higher magnification of each of the figures. White open arrowheads: cornified layer. Nucleus: blue color. Scale bars, 10 μm.

and hydrated spadefoot toad, *Scaphiopus couchi* (Hillyard, 1979; Yokota and Hillman, 1984; Viborg and Rosenkilde, 2004).

IP stimulates the increase in the synthesis of cyclic AMP *via* β-receptor (De Sousa and Grosso, 1982). This process is identical to the action of AVT in terms of stimulation of the formation of cyclic AMP. We were able to show here that IP induces the translocation of AQP-h2 and AQP-h3 proteins from the cytoplasmic pool to the apical plasma membrane, thereby increasing water permeability. This observation confirms earlier findings using freeze-fracture techniques that showed IP stimulating the movement of the intramembrane particle aggregates (Brown et al., 1983). However, our experiments provide new insight to this field in terms of elucidating the molecular mechanisms underlying the water flow across the pelvic skin of the tree frog by identifying the AQP molecules such as AQP-h2 and AQP-h3. The combined application of IP+AVT increased water permeability to levels not significantly different from AVT alone. Hillyard (Hillyard, 1979) did find an additive effect of IP and AVT as did De Sousa and Grosso (De Sousa and Grosso, 1982) with isolated skin. It is possible that there is a maximum level of water permeability given that the production of cyclic AMP is considered to reach a plateau. In comparison, water permeability did not increase in the PP (β-receptor antagonist)-treated pelvic skins or in the control skin to nearly the same degree, which is consistent with there being no evidence of translocation of AQP-h2 and AQP-h3 proteins to the apical plasma membrane. This finding suggests that AQP protein plays an important role in facilitating the permeation of water across the ventral pelvic skin by mediation *via* the β-receptor. We observed that the effect of AVT was inhibited when the pelvic skins were treated with PP+AVT combined. This inhibition may be dependent upon the physiological status of the principal cells in the FRC layer as our immunofluorescence studies indicated the presence of two cell types: the apical plasma membrane of some cells in the FRC layer is labeled while others are not, suggesting two cellular compartments that could be activated independently. In this context, De Sousa and Grosso demonstrated that the β-adrenergic response appears to be independent of AVT (De Sousa and Grosso, 1982). It is important to note that one of several β-adrenergic actions is the induction of not only translocation of AQP in the apical membrane of the principal cells but also vascular perfusion in the connective tissue underlying the epidermis (Viborg and Hillyard, 2005; Willumsen et al., 2007). Both actions stimulate the absorption of water from the pelvic skin.

In conclusion, our data demonstrate that AVT, hydrins and β-adrenergic agents stimulate water permeability *via* a common mechanism of AQP insertion into the apical membrane of the principal cells in the FRC layers. The issue of whether a V2 receptor and a β-adrenergic receptor may act independently or in different principal cells in the FRC layers remains an important question.

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REFERENCES

Acher, R., Chauvet, J. and Rouille, Y. (1997). Adaptive evolution of water homeostasis regulation inamphibians: vasotocin and hydrins. *Biol. Cell* **89**, 283-291.
Agre, P. (2006). The aquaporin water channels. *Proc. Am. Thorac. Soc.* **3**, 5-13.
Akabane, G., Ogushi, Y., Hasegawa, T., Suzuki, M. and Tanaka, S. (2007). Gene cloning and expression of an aquaporin (AQP-h3BL) in the basolateral membrane of water-permeable epithelial cells in osmoregulatory organs of the tree frog. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **292,** R2340-R2351.
Bentley, P. J. and Main, A. R. (1972). Zonal differences in permeability of the skin of some anuran Amphibia. *Am. J. Physiol.* **223**, 361-363.
Bentley, P. J. and Yorio, T. (1979). Do frogs drink? *J. Exp. Biol.* **79**, 41-46.
Brown, D., Grosso, A. and De Sousa, R. C. (1980). Isoproterenol-induced intramembrane particle aggregation and water flux in toad epidermis. *Biochim. Biophys. Acta* **596**, 158-164.
Brown, D., Grosso, A. and De Sousa, R. C. (1983). Correlation between water flow and intramembrane particle aggregates in toad epidermis. *Am. J. Physiol.* **245**, C334-C342.
De Sousa, R. C. and Grosso, A. (1982). Osmotic water flow across the abdominal skin of the toad bufo marinus: effect of vasopressin and isoprenaline. *J. Physiol.* (Lond.) **329**, 281-296.
Hasegawa, T., Tanii, H., Suzuki, M. and Tanaka, S. (2003). Regulation of water absorption in the frog skins by 2 vasotocin-dependent water-channel aquaporins, AQP-h2 and AQP-h3. *Endocrinology* **144**, 4087-4096.
Hasegawa, T., Suzuki, M. and Tanaka, S. (2005). Immunocytochemical studies on translocation of phosphorylated AQP-h2 protein in granular cells of the frog urinary bladder before and after stimulation with vasotocin. *Cell Tissue Res.* **322**, 407-415.
Hillman, S. S., Withers, P. C., Drewes, R. C. and Hillyard, S. D. (2009). In *Ecological and Environmental Physiology of Amphibians*, pp. 1-68. New York, Oxford University Press.
Hillyard, S. D. (1979). The effect of isoproterenol on the anuran water balance response. *Comp. Biochem. Physiol. C* **62C**, 93-95.
Ishibashi, K., Kuwahara, M. and Sasaki, S. (2000). Molecular biology of aquaporins. *Rev. Physiol. Biochem. Pharmacol.* **141**, 1-32.
Kohno, S., Fujime, M., Kamishima, Y. and Iguchi, T. (2004). Sexually dimorphic basal water absorption at the isolated pelvic patch of Japanese tree frog, *Hyla japonica. J. Exp. Zool. A Comp. Exp. Biol.* **301**, 428-438.
Michel, G., Ouedraogo, Y., Chauvet, J., Katz, U. and Acher, R. (1993). Differential processing of provasotocin: relative increase of hydrin 2 (vasotocinyl-Gly) in amphibians able to adapt to an arid environment. *Neuropeptides* **25**, 139-143.
Ogushi, Y., Mochida, H., Nakakura, T., Suzuki, M. and Tanaka, S. (2007). Immunocytochemical and phylogenetic analyses of an arginine vasotocin-dependent aquaporin, AQP-h2K, specifically expressed in the kidney of the tree frog, *Hyla japonica. Endocrinology* **148**, 5891-5901.
Preston, G. M., Carroll, T. P., Guggino, W. B. and Agre, P. (1992). Appearance of water channels in Xenopus oocytes expressing red cell CHIP28 protein. *Science* **256**, 385-387.
Rouille, Y., Michel, G., Chauvet, M. T. and Chauvet, J. (1989). Hydrins, hydroosmotic neurohypophysial peptides: osmoregulatory adaptation in amphibians through vasotocin precursor processing. *Proc. Natl. Acad. Sci. USA.* **86**, 5272-5275.
Takata, K., Matsuzaki, T. and Tajika, Y. (2004). Aquaporins: water channel proteins of the cell membrane. *Prog. Histochem. Cytochem.* **39**, 1-83.
Tanii, H., Hasegawa, T., Hirakawa, N., Suzuki, M. and Tanaka, S. (2002). Molecular and cellular characterization of a water channel protein, AQP-h3, specifically expressed in the frog ventral skin. *J. Membr. Biol.* **188**, 43-53.
Viborg, A. L. and Hillyard, S. D. (2005). Cutaneous blood flow and water absorption by dehydrated toads. *Physiol. Biochem. Zool.* **78**, 394-404.
Viborg, A. L. and Rosenkilde, P. (2004). Water potential receptors in the skin regulate blood perfusion in the ventral pelvic patch of toads. *Physiol. Biochem. Zool.* **77**, 39-49.
Willumsen, N. J., Viborg, A. L. and Hillyard, S. D. (2007). Vascular aspects of water uptake mechanisms in the toad skin: perfusion, diffusion, confusion. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **148**, 55-63.
Yokota, S. D. and Hillman, S. S. (1984). Adrenergic control of the anuran cutaneous hydroosmotic response. *Gen. Comp. Endocrinol.* **53**, 309-314.
Yorio, T. and Bentley, P. J. (1978). The permeability of the skin of the aquatic anuran *Xenopus laevis* (Pipidae). *J. Exp. Biol.* **72**, 285-289.