A pore-forming protein drives macropinocytosis to facilitate toad water maintaining

Zhong Zhao, Zhi-Hong Shi, Chen-Jun Ye & Yun Zhang

Maintaining water balance is a real challenge for amphibians in terrestrial environments. Our previous studies with toad Bombina maxima discovered a pore-forming protein and trefoil factor complex βγ-CAT, which is assembled under tight regulation depending on environmental cues. Here we report an unexpected role for βγ-CAT in toad water maintaining. Deletion of toad skin secretions, in which βγ-CAT is a major component, increased animal mortality under hypertonic stress. βγ-CAT was constitutively expressed in toad osmoregulatory organs, which was inducible under the variation of osmotic conditions. The protein induced and participated in macropinocytosis in vivo and in vitro. During extracellular hyperosmosis, βγ-CAT stimulated macropinocytosis to facilitate water import and enhanced exosomes release, which simultaneously regulated aquaporins distribution. Collectively, these findings uncovered that besides membrane integrated aquaporin, a secretory pore-forming protein can facilitate toad water maintaining via macropinocytosis induction and exocytosis modulation, especially in responses to osmotic stress.

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Maintaining water balance is a key challenge that amphibians face in the process of transition from water to land. In these animals, the coordinated function of the nervous, endocrine and lymphatic systems and organs involved in water-salt balance and osmoregulation (including the skin, bladder and kidneys) results in enhanced water storage and utilization and reduced evaporation. Amphibians are able to absorb water through their skin. In addition, water is reabsorbed from the tubular fluid in the kidney and from stored urine in the urinary bladder (UB). Water channel proteins aquaporins (AQPs) play key roles in transepithelial water absorption/reabsorption in these organs, and in cell volume regulation. However, it has rarely been studied on the physiological function of protein components from amphibian skin secretions in regulating of integumental water homeostasis.

Macropinocytosis is a mechanism that mediates bulk uptake and internalization of extracellular fluid and the solutes contained therein, producing endocytic vesicles with diameters of 0.2–5 μm. Macropinocytosis is an actively-dependent endocytic pathway mediated by the activation of the Ras and phosphatidylinositol 3-kinase (PI3K)-signaling pathways, which is induced by either endogenous agents such as growth factors, or by invasive microbe. This fundamental cellular process has been documented to play various patho-physiological roles in a range of normal and malignant cells, including nutrient acquisition, cell growth, traffic and renewal of membrane components, entry of pathogens, immune surveillance, and cellular motility. However, the physiological roles of macropinocytosis, a very ancient form of endocytosis, remain incompletely understood.

Pore-forming proteins (PFPs) are usually secretory proteins that exist in a water-soluble monomeric form and oligomerize to form transmembrane pores (channels). Aerolysins are bacterial β-barrel PFPs produced by *Aeromonas* species. Interestingly, numerous aerolysin family PFPs (abbreviated af-PFPs, previously referred as Aerolysin-Like Proteins, ALPs) harboring an aerolysin membrane insertion domain fused with other domains have been identified in various animals and plants. Our previous studies with the skin secretions of the toad *Bombina maxima* discovered an interaction network among af-PFPs and trefoil factors (TFFs). BmALP1, an af-PFP from the toad, can be reversibly regulated by the activation of the Ras and phosphatidylinositol 3-kinase (PI3K)-signaling pathways, which is induced by either endogenous agents such as growth factors, or by invasive microbe.

Skin secretions play pivotal roles in the physiological functions of amphibian skin. Furthermore, βγ-CAT is a major proteinaceous component of skin secretions in toad *B. maxima*. Two subunits of βγ-CAT together were estimated to account for about 50% of proteins in *B. maxima* skin secretions (Supplementary Fig. 1a). We speculated that toad skin secretions, especially βγ-CAT, might play an active role in water balance. Osmotic stress experiments were conducted to test the possible functions of βγ-CAT-containing skin secretions in maintenance of water balance in the toad. Toads *B. maxima* lost 20% of their body weight and 50% of the animals died when they were placed in the hypertonic Ringer’s solution for 24 hours (Fig. 1b and Supplementary Fig. 1b). Toad skin secretions were depleted by electro-stimulation 30 minutes before the toads were placed in different osmotic solutions. Although no toad deaths were recorded after electro-stimulation in the isotonic Ringer’s solution, mortality occurred in the hypertonic Ringer’s solution (Fig. 1b). Therefore, it appears that skin secretions are critical for the toad to cope with hypertonic environments. To further investigate the possible involvement of βγ-CAT in the toad’s water balance, qPCR was used to detect the expression of two βγ-CAT subunits in the skin, UB and kidney of toad *B. maxima* during dehydration and weight recovery (water absorption after dehydration). It was found that βγ-CAT α-subunit expression was upregulated in the skin and kidney during weight recovery via water uptake after dehydration (Fig. 1c and Supplementary Fig. 1c). In contrast, the expression of βγ-CAT β-subunit mRNA was upregulated in the toad skin during dehydration or water absorption (Fig. 1d), but not in the kidney (Supplementary Fig. 1d). Interestingly, in the UB, the mRNA levels of the both subunits were increased when toads were exposed to hypertonic Ringer’s solution, and returned to the normal level when toads were subsequently placed in isotonic Ringer’s solution (Fig. 1e, f). Therefore, βγ-CAT expression is associated with changes to external osmotic conditions.

In addition, we analyzed the expression and localization of βγ-CAT protein under the same treatment conditions via immunohistofluorescence (IHF). βγ-CAT was mainly concentrated within the epidermis and glands of the skin, and it was upregulated in the epidermis and basement membrane during osmotic stress (Fig. 1g). βγ-CAT was predominantly localized within the transitional epithelium in the UB. The expression of βγ-CAT protein in toad UB was upregulated in hypertonic Ringer’s solution and downregulated when the toad was then placed into isotonic Ringer’s solution (Fig. 1h). Collectively, these results revealed an involvement of βγ-CAT in the toad’s responses to osmotic stress.

**Results**

βγ-CAT is involved in responses to osmotic stress. Amphibian skin has played a dual role in osmoregulation during evolutionary adaptation to land environments. Accordingly, when exposed to hypertonic Ringer’s solution, toads (*B. maxima*) rapidly lost weight, which was gradually recovered when the animal was then placed into isotonic Ringer’s solution (Fig. 1a). This suggests that the toad transports water through its skin under conditions of osmotic stress.

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**Fig. 1 βγ-CAT is involved in responses to osmotic stress.**

**a** Toad weight changes were measured after placing them in isotonic, hypertonic and hypertonic/isotonic Ringer's solutions. Initial weight of toads is 19 ± 5 g (n = 5). **b** Survival rates of toads in isotonic (i) and hypertonic (h) Ringer's solution were determined after 48 hours. Toads were placed in each of the solutions after 30 minutes with (+) or without (-) electro-stimulation to delete toad skin secretions (n = 6). **c-f** The expression of βγ-CAT subunits in toad skin and UB was analyzed by real-time fluorescent quantitative PCR (n = 6). Toads were placed in isotonic or hypertonic Ringer's solution for 3 hours before the samples were collected. In the hypertonic/isotonic group, the toads were first placed in the hypertonic solution for 3 hours, then moved to isotonic solution for a further 3 hours before the samples were collected. **g, h** Following placement of toads in isotonic, hypertonic or hypertonic/isotonic Ringer's solution as described above, the localization and expression of βγ-CAT in the toad skin (**g**) and UB (**h**) tissues were analyzed by immunohistofluorescence (IHF). Ep epidermis, De dermis, Mg mucous gland, Gg granular gland, Lu lumen, Te transitional epithelium. Scale bars, 200 μm. *P < 0.05 and **P < 0.01 by the Gehan-Breslow-Wilcoxon test in survival rate analysis. ns (P ≥ 0.05); *P < 0.05, **P < 0.01 and ***P < 0.001 by unpaired t test in other experiments. All data represent the mean ± SD and are representative of at least two independent experiments. See also supplementary Fig. 1.
βγ-CAT counteracts cell dehydration under extracellular hyperosmosis. βγ-CAT is a vital functional protein of toad *B. maxima*, and its constitutive expression can be detected in various toad tissues, including skin secretions and epithelial cells from the skin and UB, as well as kidney and peritoneal cells. Enogenous secretion of βγ-CAT in these toad-derived cells was further analyzed by a hemolysis assay, a sensitive method to detect the presence of biologically active βγ-CAT. All the media of these cultured toad cells showed potent hemolysis activity, which was totally inhibited by anti-βγ-CAT antibodies, confirming the presence of secreted βγ-CAT (Supplementary Fig. 2a). In addition, we measured the cytotoxicity of βγ-CAT to *B. maxima* cells. Previously, it was reported that βγ-CAT showed no cytotoxicity to toad peritoneal cells at dosages up to 400 nM. The present study further determined that cytotoxicity of βγ-CAT to skin and UB epithelial cells and kidney cells of *B. maxima* occurred only when its concentration reached 2 μM (Supplementary Fig. 2b), a level much higher than physiological concentrations (20–100 nM), as determined in the toad peritoneum. In contrast, mammalian cells were much more sensitive to βγ-CAT. Although βγ-CAT showed no cytotoxicity to MDCK, Caco-2 and T24 cells at 10 nM, the protein caused cell death when dosages used were higher than 50–100 nM (Supplementary Fig. 2c). Thus, in all subsequent experiments concerning the addition of purified βγ-CAT to cells, the protein dosages used were: epithelial cells from toad skin, 100 nM; UB epithelial cells, 50 nM; peritoneal cells, 50 nM; mammalian MDCK, 10 nM; Caco-2, 10 nM; and T24, 5 nM.

To further explore the potential role of βγ-CAT in water transport, we investigated whether the protein is able to counteract cell dehydration under extracellular hyperosmosis. We confirmed the action of βγ-CAT on toad UB epithelial cells, mammalian MDCK, Caco-2 and T24 cells, as determined by the formation of βγ-CAT oligomers after treatment with the protein (Fig. 2a and Supplementary Fig. 2d). βγ-CAT oligomers were detected in toad UB epithelial cells without the addition of the protein, possibly caused by endogenously secreted βγ-CAT (Fig. 2a). The addition of purified βγ-CAT further increased the concentration of oligomers of the protein in the UB epithelial cells (Fig. 2a). Then, we analyzed the electrophysiological characteristics of βγ-CAT on outside-out patches of HEK293 cells. βγ-CAT-induced macroscopic currents were recorded and normalized, which has the characteristic of inward rectification (Fig. 2b). These proved that βγ-CAT could form transmembrane pores (channels) and mediate ion flow after oligomerization on the membrane. We further investigated ion selectivity of βγ-CAT channels through ion replacement experiments. Asymmetric 150:15 mM NaCl solutions left-shifted the reversal potential from 0 mV to −61.2 mV, which is very close to the theoretical equilibrium potential of Na⁺, indicating that the βγ-CAT channels are permeable to Na⁺ but not to Cl⁻ (Fig. 2c). These results demonstrated that the βγ-CAT channels allowed Na⁺ flow. On this basis, we explored the potential role of βγ-CAT when cells were challenged by extracellular hyperosmosis using hypertonic solution. We observed that βγ-CAT promoted volume recovery of MDCK, Caco-2 and T24 cells in hypertonic solution (Fig. 2d), revealing the capacity of the protein to facilitate water uptake under hyposmotic conditions. In consistency with those observations, immune-depletion of endogenous βγ-CAT further decreased the cell diameters of toad UB epithelial cells in hypertonic Ringer’s solution relative to those in the absence of anti-βγ-CAT antibodies (Fig. 2e). It is well documented that AQP5s can mediate the rapid cellular flow of water. Thus, it is necessary to clarify whether the cell volume recovery by water uptake mediated by βγ-CAT under osmotic stress was associated with AQP function. Because the mercury-sensitive sites of B. maxima AQP5s (BmAQPs) in the UB were evolutionarily conserved (Supplementary Fig. 2e, f), HgCl₂ was used to inhibit BmAQPs function. Cell volume was unchanged in hypertonic Ringer’s solution after blocking BmAQPs with HgCl₂. Conversely, cell volume recovery of toad UB epithelial cells via water uptake was clearly reduced after immunodepletion of endogenous βγ-CAT (Fig. 2f). Taken together, these results revealed the capacity of βγ-CAT to promote cell volume recovery by stimulating water acquisition during extracellular hyperosmosis, and indicated that the effect was independent of water flow via plasma membrane AQP5s.

βγ-CAT promotes macropinocytosis. βγ-CAT participates in cell volume regulation, suggesting its involvement in water transport. We next analyzed the possible cellular mechanism behind this phenomenon. Water acquisition can be rapidly realized through AQP5s and/or macropinocytosis. Previously, βγ-CAT-enhanced pinocytosis in a murine dendritic cell (DC) model. In the present study, we carefully studied the ability of βγ-CAT to stimulate and participate in macropinocytosis in various types of *B. maxima* cells. First, immunoelectron microscopy (IEM) revealed that βγ-CAT was localized in cellular pseudopodia and macropinosomes with diameters up to 300 nm formed by macropinocytosis in toad skin and UB tissues (Fig. 3a). βγ-CAT was also present in the intercellular spaces of epithelial cells (Fig. 3a). 70-kDa dextran is a marker for macropinocytosis, while Lucifer Yellow (LY) can be used as a marker of fluid-phase endocytosis including macropinocytosis. In epithelial cells obtained from toad skin and UB, and in toad kidney or peritoneal cells, immunodepletion of endogenous βγ-CAT by anti-βγ-CAT antibodies greatly decreased internalization of LY and FITC-dextran, relative to cells observed in the presence of control rabbit IgG (Fig. 3b, c and Supplementary Fig. 3a, b). Surprisingly, exogenously added control rabbit IgG can induce pinocytosis. The widespread presence of IgG receptors (e.g., FcRn) in cells may contribute part of the effect. In fact, the FcRn-like gene of *B. maxima* was also present in toad cells (Supplementary Fig. 3c). For unknown reasons, rabbit IgG contributes to pinocytosis for toad cells as an exogenous protein. We found that control rabbit IgG and anti-βγ-CAT rabbit-derived antibodies were equally potent in inducing macropinocytosis in MDCK cells (Supplementary Fig. 3d). Therefore, these demonstrated that anti-βγ-CAT rabbit-derived antibodies can reduce βγ-CAT-induced macropinocytosis by immunodepletion of endogenous βγ-CAT. Furthermore, addition of βγ-CAT to cells from toad skin and UB, and mammalian MDCK and T24 cells enhanced the internalization of LY and FITC-dextran (Fig. 3d, e and Supplementary Fig. 3e, f). We found that βγ-CAT was endocytosed and located in the macropinosomes of MDCK cells (Supplementary Fig. 3g). In addition, total Na⁺ concentrations in toad UB epithelial cells and MDCK cells treatment with βγ-CAT were 3.5 and 1.1 times of that of the vehicle group, respectively (Fig. 3f).

Next, the molecular mechanism of macropinocytosis induced by βγ-CAT was investigated using pharmacological agents. 5-(N-ethyl-N-isopropyl) amiloride (EIPA, a Na⁺/H⁺-exchanger inhibitor) and wortmannin (WORT, a PI3K inhibitor) are commonly used to inhibit macropinocytosis. We found that EIPA and WORT reduced macropinocytosis induced by βγ-CAT in toad UB epithelial cells and mammalian MDCK cells (Fig. 3g, h). Moreover, βγ-CAT stimulated the phosphorylation of Akt and PI3K and the expression of Rac1L23 in MDCK cells (Fig. 3i). Taken together, these results demonstrated that βγ-CAT, an endogenously secreted complex of a PFP and a TFF, stimulates PI3K and the expression of Rac1 in MDCK cells (Fig. 3i).

Thus, it is necessary to clarify whether the cell volume recovery by water uptake mediated by βγ-CAT under osmotic stress was associated with AQP function. Because the mercury-sensitive sites of B. maxima as well as in various mammalian cells.
βγ-CAT in AQP regulation. AQPs and ion flux are involved in water transport and volume regulation of toad UB cells under stimulation by multiple hormones. Internalization by macropinocytosis is unselective, whereas transitional epithelial cells are polar. Therefore, we assessed the relationship between βγ-CAT-stimulated macropinocytosis and BmAQP2. We observed that the location of βγ-CAT and BmAQP2 in toad UB epithelial cells differed between isotonic, hypertonic and hypertonic/isotonic (hypertonic followed by returning to isotonic) Ringer’s solutions (Fig. 4a). When toads were exposed to isotonic Ringer’s solution, βγ-CAT and BmAQP2 were located together and were distributed on both the apical and basal sides of the transitional epithelium in toad UB. In hypertonic Ringer’s solution, βγ-CAT and BmAQP2 shifted their location to the basal or lateral sides of the UB transitional epithelium. In contrast, when the toads were returned to isotonic Ringer’s solution from hypertonic Ringer’s solution, βγ-CAT and BmAQP2 began to migrate to the apical side of the transitional epithelium. This observation suggests that macropinocytosis induced by βγ-CAT is involved in the internalization and transport of BmAQP2 in the UB. In MDCK, intracellular colocalization of βγ-CAT and AQP2 was also observed (Fig. 4b). These results indicated that macropinocytosis stimulated by βγ-CAT plays a role in the regulation of AQPs under various osmotic conditions, revealing another aspect of this protein in the modulation of toad water balance.

βγ-CAT enhances exosome release. The secretion of exosomes is an important aspect of cell exocytosis that can be viewed as a means of selective transport of materials among cells and a mode of intercellular communications. Because βγ-CAT promotes...
macropinocytosis, we further explored the cellular fate and role of βγ-CAT-containing vesicles. βγ-CAT was detected in both multivesicular bodies (MVBs) and intraluminal vesicles (ILVs) of UB epithelial cells (Fig. 5a). Considering that 98% of toad UB epithelial cells survived 3 hours in vitro culture at room temperature (Supplementary Fig. 4a), exosomes secreted by these cells were collected and observed (Fig. 5b), which contained typical exosome markers CD63, TSG101 and flotillin-1, and the number of exosomes was increased when these cells were treated with purified βγ-CAT (Fig. 5c). Nanoparticle tracking analysis (NTA) revealed that immunodepletion of endogenous βγ-CAT attenuated exosome release from toad UB epithelial cells and peritoneal cells (Fig. 5d and Supplementary Fig. 4b), while the addition of βγ-CAT substantially augmented exosome release from these cells...
Fig. 3 βγ-CAT promotes macropinocytosis. a Ultrastructural localization of βγ-CAT in toad skin and UB tissues as analyzed by IEM. Vehicle (rabbit IgG control). Endocytic vesicles formed by macropinocytosis (black asterisks), and distribution of βγ-CAT on vesicles (red arrows) and intercellular spaces (red triangles). b, c Immunodepletion of endogenous βγ-CAT decreased macropinocytosis. Toad skin (b) and UB (c) epithelial cells were incubated with 50 μg mL⁻¹ anti-βγ-CAT antibody to immunodeplete endogenous βγ-CAT for 30 min. The mean fluorescence intensity was determined by flow cytometry with 100 μg mL⁻¹ of 70 kDa FITC-label dextran and Lucifer Yellow (LY) for 30 minutes. Rabbit IgG (antibody control). Vehicle (antibody absent control). d, e The addition of purified βγ-CAT augmented macropinocytosis. The mean fluorescence intensity of LY and FITC-label dextran in toad skin (d) and UB (e) epithelial cells was determined by flow cytometry with or without additional 100 nM or 50 nM βγ-CAT, respectively. f Fold changes of [Na⁺] in toad UB epithelial cells (n = 6) and MDCK cells (n = 8) with and without the addition of 50 nM or 10 nM βγ-CAT for 3 hours, respectively. g, h The effect of inhibitors on macropinocytosis induced by βγ-CAT. Toad UB epithelial cells (g) and MDCK cells (h) were incubated with and without 100 μM EIPA or 20 μM WORT for 1 hour. Then the cells were cultured with 100 μg mL⁻¹ FITC-label dextran with 50 nM (toad UB cells) or 10 nM (MDCK cells) βγ-CAT for 30 minutes. Rac123 and phosphorylation of Akt and P38 in response to 10 nM βγ-CAT in MDCK cells for 15, 30 or 60 minutes as determined by Western blotting and bands were semiquantified with ImageJ. The black dotted line refers to the blank control, and data represent the mean ± SD of triplicate samples in b, e, g, h. Data represent the mean ± SD of at least three independent replicates in f and i (P ≤ 0.05), *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001 by the unpaired t test. All data are representative of at least two independent experiments. See also supplementary Fig. 3.

Fig. 4 βγ-CAT in AQP regulation. a Colocalization of βγ-CAT and BmAQP2 in the UB tissue of toad B. maxima after toads were placed in isotonic, hypertonic or hypertonic/isotonic Ringer’s solution for 3 hours as analyzed by immunohistoﬂuorescence. Scale bars, 25 μm. b Intracellular colocalization of βγ-CAT and AQP2 in MDCK cells with or without the treatment of 10 nM βγ-CAT for 15 minutes as determined by immunohistoﬂuorescence. Scale bars, 30 μm. All data are representative of at least two independent experiments.

(Fig. 5e and Supplementary Fig. 4c). None of these treatments changed the average exosome diameter. Similar results were also obtained in mammalian MDCK and T24 cells (Supplementary Fig. 4d, e). Collectively, these results showed that βγ-CAT promotes the production and release of exosomes in both toad and mammalian cells.

We further investigated the properties of exosomes stimulated by βγ-CAT. The size distribution of exosomes derived from toad UB epithelial cells was determined by flow cytometry for nanoparticle analysis. The diameters of these exosomes were mainly concentrated in the range of 50–150 nm (Supplementary Fig. 4f). When 1 mg mL⁻¹ of FITC-dextran was incubated with isolated exosomes, the exosomes did not contain FITC-dextran (Supplementary Fig. 4g). In contrast, when dextran was added to the culture of toad UB epithelial cells under the same conditions, FITC-dextran was identified in exosomes secreted by the cells (Supplementary Fig. 4g). This observation indicated that isolated exosomes did not take up 70-kDa dextran, but dextran was taken up by toad UB epithelial cells by macropinocytosis and released from cells in the form of exosomes. Interestingly, the proportion of exosomes containing FITC-dextran remained unchanged in toad UB epithelial cells with or without the addition of purified βγ-CAT (Fig. 5f), and there was no difference in the mean fluorescence intensity of these exosomes (Fig. 5g). These results suggested that βγ-CAT promotes exocytosis and facilitates the transcellular transport of extracellular substances like dextran by increasing exosome release. However, the apparent properties of exosomes released seem not altered as assayed at the present stage.

Both BmAQP2 and βγ-CAT oligomers were detected by Western blotting in exosomes released from toad UB epithelial cells, reflecting the presence of endogenous βγ-CAT. The addition of βγ-CAT to cells greatly enhanced the quantity of BmAQP2 and βγ-CAT oligomers detected (Fig. 5h). IEM demonstrated colocalization of BmAQP2 and βγ-CAT in individual exosomes (Fig. 5i). These observations suggested that βγ-CAT drives the extracellular recycling and/or intercellular communication of AQPs via exosome release.

Furthermore, we analyzed the effect of βγ-CAT on Na⁺ levels in exosomes from MDCK cells. No difference was observed for total Na⁺ concentrations in exosomes produced by the same number of cells treated with or without 10 nM βγ-CAT (Fig. 5j). However, βγ-CAT increased exosome release of MDCK by 6.8 times (Supplementary Fig. 4d), so the mean Na⁺ concentrations of exosomes were reduced (Fig. 5k). In addition, an increase in total Na⁺ concentrations in exosomes induced by βγ-CAT were found in hypertonic media under the same conditions (Fig. 5l).
Amphibians live both on land and in water, and their skin is naked and acts as an important organ in water balance, respiration (gas exchange) and immune defense. The PFP complex βγ-CAT is widely expressed in B. maxima water balance organs, including skin, UB and kidney (Fig. 1c–h, and Supplementary Fig. 1c, 1d). Previous studies indicated the expression of βγ-CAT in various B. maxima tissues. Interestingly, in contrast to its potent cytotoxicity to various mammalian cells, βγ-CAT only shows toxic effects in toad cells in a dosage up to 2 μM (Supplementary Fig. 2b), which is much higher than βγ-CAT physiological concentrations observed.
exogenous PFP protein, which are normally present in the toad18,21. These observations support the notion that the PFP complex βγ-CAT can not be simply viewed as a cell death inducer and/or a microbicide, and besides its roles in immune defense22–26, the protein exhibits other essential physiological functions in this toad.

B. maxima skin is covered with skin secretions containing various biological molecules that fulfill a range of physiological function, including hormone-like peptides, antimicrobial peptides, α-FPfs, TFFs, and haem b-containing albumin16,40,41. Indeed, our results revealed that the skin secretions are necessary to prevent dehydration (Fig. 1b). We further demonstrated that a major skin secretion component βγ-CAT has a role in toad water homeostasis in osmoregulatory organs. Although most amphibians do not live in hypertonic environments and their skin is rarely challenged by extreme water loss, the kidney and UB are subject to a variety of hypertonic challenges under physiological conditions such as urine formation and concentration. In fact, the differential expression of βγ-CAT in UB with osmotic environment suggests that βγ-CAT is involved in the dynamic change of UB water transport (Fig. 1e, f and Fig. 4a). Therefore, we further demonstrated that βγ-CAT has a role in toad water homeostasis with toad UB cells and mammalian kidney cells. This PFP complex possesses the capacity to stimulate and participate in macropinocytosis (import) and exosome release (export) by epithelial cells of toad osmoregulatory organs, which promotes water and Na+ transport involved in water homeostasis. It should be emphasized that since B. maxima is a non-model species, it is currently difficult to further explore the physiological effects of βγ-CAT at the animal level by means of gene knockout.

Previous studies with mammalian cells and toad peritoneal cells showed that βγ-CAT exerts its biological actions by endocytosis and the formation of channels on endolysosomes17,21–24, but the endocytic pathway of the protein remains elusive. Macropinocytosis, also referred to as cell drinking, is a form of endocytosis that mediates the non-selective uptake of extracellular fluid and solutes6,12. In a murine DC model, βγ-CAT increases the internalization of ovalbumin (antigen), presumed to be mediated by enhanced macropinocytosis24. The present study carried out on various toad-derived cells, including epithelial cells from osmoregulatory organs and peritoneal cells, clearly showed that βγ-CAT is capable of inducing and participating in macropinocytosis. Macropinocytosis stimulated by βγ-CAT has been shown to facilitate the entry of water and Na+ into cells as assayed in epithelial cells from toad osmoregulatory organs (Fig. 2f and Fig. 3f). This may explain the role of βγ-CAT in promoting the recovery of cell volume under hypertonic stress (Fig. 2d–i). Given that IgG binds widely distributed IgG Fc receptors to induce pinocytosis32, such as MHC-class-I-like IgG receptor protein FcRn, the co-presence of non-specific exogenous rabbit IgG and active βγ-CAT may lead to an increase in macropinocytosis (Fig. 3b, c and Supplementary Fig. 3a, b). Growth factor-induced macropinocytosis is mediated by the activation of the Ras and PI3-kinase signaling pathways11. Similarly, activation of PI3-kinase and Akt signaling was involved in macropinocytosis stimulated by βγ-CAT, as suggested by the effects of pharmacological inhibitors and the phosphorylation analysis of PI3K and Akt (Fig. 3g–i). However, in contrast to classic growth factors, which bind to membrane protein receptors to initiate their signaling, βγ-CAT targets gangliosides and sulfatides as receptors in lipid rafts to initiate its cellular effects21. The signals downstream from these lipid components and their relationship to the induction of macropinocytosis are presently unclear. In this respect, possible differences between macropinocytosis stimulated by growth factors and that induced by βγ-CAT should be investigated.

While engulfing large volumes of fluid, macropinocytosis also internalizes cell surface proteins such as receptors and integrins42,43. It has been proposed that endocytosis stimulated by βγ-CAT plays a role in the sorting of specific plasma membrane elements, such as functional integrated proteins or lipid components, which regulate cell responses to environmental variations17. In addition, internalization and recycling of AQPs between the plasma membrane and the endosomal compartment have roles in controlling water uptake and conservation5,28. Accordingly, colocalization of βγ-CAT with AQPs was observed in endocytic organelles (Fig. 4), suggesting macropinocytosis induced by this PFP protein drives endocytosis and recycling of AQPs. Clearly, the presence of AQPs in the plasma membrane might facilitate water loss when cells faced by hypertonic osmotic stress, thus internalization of AQPs induced by βγ-CAT could counteract dehydration by preventing rapid water efflux and cell shrinkage.

The secretion of extracellular vesicles comprising exosomes and microvesicles represents a novel mode of intercellular communication and material exchange38,44. Previously, βγ-CAT was found to augment βγ-CAT-containing exosome release from murine DCs, which activates T cell response effectively24. Because βγ-CAT is a factor exogenous to murine DCs, the explanation of the phenomenon is not obvious. However, the PFP is an endogenous element to toad cells. The present study illustrated that cell exocytosis in the form of exosome release was indeed augmented in the presence of βγ-CAT, as determined in diverse toad-derived cells (Fig. 5d, e and Supplementary Fig. 4b, c), revealing that mediation of cell exocytosis via exosome release is an intrinsic property of this PFP protein. The FcRn expressed by many cells, which can promote exocytosis and recycling of IgG45. The exocytosis effect of βγ-CAT may be further amplified by
exocytosis and circulation of IgG (Fig. 5d and Supplementary Fig. 4b). Previous studies demonstrated that βγ-CAT characteristically neutralizes the acidification of endocytic organelles containing this protein22-24. This cellular process may result in the transformation of βγ-CAT-containing endocytic organelles into MVBs that does not fuse with lysosomes for the degradation of contained solutes. The presence of extracellular dextran as a βγ-CAT-induced tracer of endocytosis in βγ-CAT-containing exosomes also supports the above view (Fig. 5f, g).

The observation that βγ-CAT both stimulates and participates in macropinocytosis and exosome release strongly implies that this PFP is involved in transcellular transport of internalized extracellular substances by PFP-driven cellular import and export in vesicular forms through endolysosomal pathways. This property is particularly important in vivo, where βγ-CAT could transport external substances such as water and Na+ to internal environments of toad tissues without disruption of the cellular tight junctions that maintain epithelial barrier functions. Interestingly, AQPs were identified in exosomes released in the presence of βγ-CAT (Fig. 5h, i), suggesting that βγ-CAT modulates water homeostasis by driving extracellular and/or intercellular communication of these water channels. Alternatively, βγ-CAT-mediated exocytosis might function as a means for expulsion of noxious and indigestible solutes contained in fluid macropinocytosed by cells for water acquisition, like the expelling of dextran from toad UB epithelial cells (Fig. 5f, g). The role of βγ-CAT in expelling noxious substances engulged by macropinocytosis via exocytosis induction and the cellular sorting mechanism involved are important future challenges.

Our study does not rule out the potential involvement of other physiological water transport mechanisms, such as AQPs and ion channels. In fact, our findings support the possibility that βγ-CAT may play an important role in toad *B. maxima* by participating in the regulation of classical water transport mechanisms, especially in the face of osmotic stress. It is generally understood that classic membrane integrated proteins such as AQPs and ion channels play fundamental roles in water transport and homeostasis. Furthermore, the tight junction protein claudin-2 mediates the permeability to transepithelial water movements under osmotic or Na+ gradients through the paracellular pathway. The present study has introduced a new player, a SELC protein βγ-CAT, into the network of water transport and homeostasis via the modulation of cellular material import and export through endolysosomal pathways. A cellular working model of the PFP βγ-CAT in promoting water uptake and maintaining was proposed in toad *B. maxima* (Fig. 6). In this model, water and Na+ can be imported by macropinocytosis induced by the PFP βγ-CAT. Meanwhile, the transmembrane channel formed by βγ-CAT can mediate the Na+ flow (Fig. 2b, c). Previous studies have shown that the channel formed by βγ-CAT can cause cation ion flux. Unusually, the average Na+ concentrations of βγ-CAT-containing exosomes were substantially lower than those of the control (Fig. 5j, k). This phenomenon suggested that the presence of channels formed by βγ-CAT can lead to rapid efflux of Na+ out of endolysosomes and/or exosomes. Thus, Na+ can be release into cytosol through βγ-CAT channels in endocytic organelles, which drives water release to the cytosol via AQPs (Fig. 4). This is similar to the situation of two-pore channels working in mammalian macrophages. Enhanced exosome release mediated by βγ-CAT may promote the export of ions such as Na+ (Fig. 5l), and/or water, into the intercellular spaces below tight junctions, favoring water absorption into deeper cell environments. Meanwhile, membrane-integrated ion channels and AQPs in the basal plasma membrane of epithelial cells could mediate the efflux of Na+ and water into internal tissues underneath these cells achieving the absorption/reabsorption of water into toad internal tissues. Drinking water by macropinocytosis is energetically expensive. However, the function of secretory PFP βγ-CAT in water acquisition and maintaining is necessary in light of various osmotic conditions that toad *B. maxima* has to face throughout its life cycle.

Secretory PFPs have been identified in organisms from all kingdoms of life and, af-PFPs are widely distributed in plants and animals. Based on a wealth of experimental evidence on the af-PFP and TFF complex βγ-CAT from toad *B. maxima*; we have proposed the hypothesis of the SELC pathway mediated by a PFP, which drives cellular material import and export through endolysosomal pathways. The present study provides further functional evidence to support our previous hypothesis. Regulated relying on environmental cues, SELC protein βγ-CAT has been shown to promote cellular intake and transport of environmental materials, like antigens, water with ions and plasma membrane components (see reference 24 and present study) relying on distinct cell contexts and surrounding, representing new entry points for cell vesicular delivery systems. These capacities raise the possibility that besides working in antigen presentation and water maintaining, βγ-CAT and/or its homologues could play active and fundamental roles in cell nutrient acquisition and metabolism flexibility, as previously proposed, which are intriguing subjects for further investigation. These PFPs and classic membrane integrated proteins (like solute carrier family members) can form two coordinated cell arms in extracellular nutrient sensing, sampling and acquiring. Obviously, these PFPs acting as SELCs should be essential to cells in those cases that classic membrane solute carriers are blockaded or even absent. Our findings will serve as clues for uncovering novel cellular strategies and the physiological roles of PFPs on material import and export via endolysosomal systems among cells and environments in living organisms, especially those in mammals.

In conclusion, the current work elucidated an unexpected role of a secretory PFP in water transport and homeostasis. βγ-CAT, an af-PFP and a TFF complex, induced and participated in macropinocytosis and exosome release in epithelial cells from *B. maxima* osmoregulatory organs. These effects could explain the role of the PFP complex in promoting the internalization, transport and release of water, Na+ and AQPs, working together in the maintenance of toad cell volume regulation and water homeostasis (Fig. 6). Together with membrane-integrated AQPs and ion channels, the action of secretory βγ-CAT can promote the overall achievement of water maintaining and balance, especially when the toad encounters hypertonic osmotic stress.

**Methods**

**Animals.** Toads (*B. maxima*) were captured in the wild and raised at room temperature by feeding with live *Tenebrio molitor*. Toads with an average weight of 19 ± 3 g were used in experiments after fasting in isotonic Ringer’s solution for 3 days. All the procedures and the care and handling of the animals were approved by the Institutional Animal Care and Use Committee at Kunming Institute of Zoology, Chinese Academy of Sciences (Approval ID: IACUC-OE-2021-05-001).

**Animal experiment.** Formulation of isotonic Ringer’s solution (111.2 mM NaCl, 1.9 mM KCl, 1.1 mM CaCl2, 2.4 mM NaHCO3, 1.6 mM MgCl2) was modified based on a previous report. After fasting, toads in the hypertonic group were placed in hypertonic Ringer’s solution (*Ringer’s solution with 222.4 mM NaCl*) for 3 hours and their weight changes were recorded. Toads in the hypertonic/isotonic group were then transferred from hypertonic Ringer’s solution to isotonic Ringer’s solution for 3 hours and their weight changes were recorded. Weight changes of toads in isotonic Ringer’s solution were recorded for 6 hours as a reference, termed the isotonic group. The skin of some toads was electrically stimulated (4.5 V DC, pulse duration 10 ms) for 3 minutes to deplete skin secretions, and toads were placed in isotonic Ringer’s solution for 30 minutes. Their survival rates were then recorded after 48 hours in isotonic and hypertonic solutions.
Cell culture. Toad epithelial cells were obtained by tissue digestion. Specifically, the UB, skin, and kidney tissues of *B. maxima* were dissected from five toads whose spinal cord had been destroyed. Tissues were further rinsed and stripped in Ringer’s solution to remove residual blood, mucus and other impurities. They were then cut into pieces and washed twice in Ringer’s solution, followed by oscillatory digestion with trypsin at room temperature for 40 minutes. The cells were separated with a 200-mesh sieve into a 50 mL centrifuge tube (Jet Biofl, Cat CFT011500). In addition, toad peritoneal cells were extracted from the peritoneal cavity of toads using a 2,000 rpm for 5 min at 4 °C. The digestive cells in toad skin and UB tissues contain around 90% and 60% epidermal cells, respectively, as analyzed by flow cytometry using an anti-pan-cytokeratin AE1/AE3 monoclonal antibody (Invitrogen, Cat 53-9003-80).

Madin-Darby canine kidney (MDCK), Caco-2, HEK293 and T24 cell lines were purchased from Kunming Cell Bank, Chinese Academy of Sciences. The cells were cultured in DMEM/F-12 (Biological Industries, Cat 01-172-1 A) containing 10% fetal bovine serum (Biological Industries, Cat 04-001-1 A) and 1% levooxacin (Sigma, Cat L9655).

**Purification of βγ-CAT.** βγ-CAT purification and purity analysis were carried out according to the previous description. Briefly, digested MDCK, Caco-2 and T24 cells were cultured in serum-free medium for 30 minutes at a concentration of 2×10^6 cells per mL before detection. To determine cell diameters, we treated MDCK, Caco-2 and T24 cells with hypertonic PBS (adding NaCl to 0.01 M PBS increased osmotic pressure to 400 mOsm) containing purified βγ-CAT at doses of 10 nM, 10 nM and 5 nM, respectively. Toad UB epithelial cells were incubated with or without 0.3 mM HgCl2 for 10 minutes at a concentration of 2×10^6 cells per mL after incubating with 50 μg mL^-1 rabbit-derived anti-βγ-CAT antibodies for 30 minutes at room temperature. Diameter changes of toad UB epithelial cells were detected in isotonic or hypertonic Ringer’s solution. The final concentration of all cells was 1×10^6 cells per mL. The average cell diameter was determined by a Countstar Automated Cell Counter (ALIT Life Science, Shanghai, China).

**Cell viability assay.** The MTS assay was used to detect the cytotoxicity of βγ-CAT. In brief, MDCK and T24 cell lines were seeded in 96-well plates at 1×10^4 cells per well and cultured overnight at 37 °C in 5% CO₂. Cells were incubated with the MTS reagent (Promega, Cat G3580) in the dark for 1 hour after treatment with βγ-CAT at room temperature for 2 hours. The absorbance of the cell culture supernatant was measured with a 2,000 rpm for 5 min. The absorbance was measured at 490 nm with the Infinite 200 Pro microplate reader (Tecan, Männedorf, Switzerland).

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**Cell diameter measurement.** Previously described methods were modified as appropriate. Briefly, digested MDCK, Caco-2 and T24 cells were cultured in serum-free medium for 30 minutes at a concentration of 2×10^6 cells per mL before detection. To determine cell diameters, we treated MDCK, Caco-2 and T24 cells with hypertonic PBS (adding NaCl to 0.01 M PBS increased osmotic pressure to 400 mOsm) containing purified βγ-CAT at doses of 10 nM, 10 nM and 5 nM, respectively. Toad UB epithelial cells were incubated with or without 0.3 mM HgCl2 for 10 minutes at a concentration of 2×10^6 cells per mL after incubating with 50 μg mL^-1 rabbit-derived anti-βγ-CAT antibodies for 30 minutes at room temperature. Diameter changes of toad UB epithelial cells were detected in isotonic or hypertonic Ringer’s solution. The final concentration of all cells was 1×10^6 cells per mL. The average cell diameter was determined by a Countstar Automated Cell Counter (ALIT Life Science, Shanghai, China).

**Quantitative real-time PCR.** The mRNA levels of βγ-CATα and βγ-CATβ in toad skin, UB and kidney were detected by qRT-PCR using a HiFi qPCR SYBR Green Master Mix (No Rox) kit (Yeasen, Cat 11201ES03). The cycle counts of target genes were normalized to those of β-actin. The mRNA levels of FcRn in toad cells from skin, UB and kidney were detected by RT-PCR. Primer sequences in this study are shown in Supplementary Table 1.

**RBITC-labeling of βγ-CAT.** The labeling reactions were performed in accordance with a previously described procedure with some modifications. Purified βγ-CAT was dialyzed overnight in a cross-linking solution (NaHCO3 7.36 g, Na2CO3 1.06 g, and NaCl 7.36 g dissolved in 1 L of water; pH 9.0) using a 3,500 Da dialysis membrane (Biosharp, Cat BS-QT-021), and 1 mg of RBITC (Sigma, Cat 283924) was dissolved in 1 mL of DMSO (Sigma, Cat D2650). The RBITC solution was slowly added to βγ-CAT to give βγ-CAT: RBITC = 1 mg:150 μg and the sample was incubated overnight at 4 °C. The sample was then incubated in PBS for 2 hours at 37 °C. The labeled samples were then dialyzed against 1 mL of PBS at 4 °C for 2 hours. The labeled samples were then dialyzed against 1 mg mL^-1 with PBS and stored at 4 °C in the dark.
Isolation of exosomes. The method to isolate exosomes was optimized based on a previous description. Briefly, MDCC and T24 cells were grown to 90% on 100-mm cell culture dishes. The cells were cultured in twenty cell culture dishes with and without 10 nM or 5 nM βγ-CAT for 3 hours at 37 °C. Cell supernatant (200 μL) was collected for gradient centrifugation (300 x g for 30 minutes; 500 x g for 30 minutes; 10,000 x g for 40 minutes) at 4 °C to remove residual cells, debris and microvesicles. Exosomes were obtained by ultracentrifugation at 100,000 x g with rotor P100AT2 for 2 hours at 4 °C, and then resuspended in PBS, and enriched again in the CP100WX preparative ultracentrifuge (HATACHI, Tokyo, Japan). The enriched exosomes were stored at −80 °C for follow-up experiments.

Flow cytometry. 2×10^6 MDCC and T24 cells were incubated with 100 μg/mL−1 70 kDa FITC-label dextran (Sigma, Cat G6945) or Lucifer Yellow (Sigma, Cat L0144) in the dark at 37 °C for 30 minutes with and without 10 nM or 5 nM βγ-CAT, respectively. The samples were followed by fluorescence detection of FITC or Lucifer Yellow was recorded using a Zeiss LSM 880 microscope system (Carl Zeiss, Oberkochen, Germany).

Transmission electron microscopy (TEM) and immunoelectron microscopy (IEM). The experiments were based on the previous report. Tissue samples were fixed overnight with 0.1 M PB (pH 7.4) containing 3% paraformaldehyde 0.1% glutaraldehyde at 4 °C, washed with 0.1 M PB four times for 15 minutes, and then washed with 0.1 M glycine in 0.1 M PB for 30 minutes at 4 °C. After ethanol gradient dehydration, the sample was embedded in LR white resin (Sigma, Cat L9774) and polymerized at 55 °C for 24 hours. 100-nm ultrathin sections were prepared using an EM UC7 ultramicrotome (Leica Microsystems, Wetzlar, Germany) and loaded onto 200-μm Ni grids (EMCN, Cat BZI0262Na). Enriched exosomes were directly attached to Ni grids for 10 minutes before experimental treatment. The samples were washed with deionized water for 2 minutes and then blocked with 1% BSA for 5 minutes. After overnight incubation with mouse-derived anti-βγ-CAT or rabbit-derived anti-AQP2 (ImmunoWay, Cat YT0290) primary antibodies at 4 °C, the samples were washed with deionized water 10 times. Then, the samples were incubated with 5-nm or 10-nm colloidal gold-conjugated secondary antibody (Sigma, Cat G7527 and G7402) at room temperature for 2 hours, and washed 10 times at room temperature. Exosomes were stained with 1% uranyl acetate for 7 minutes and lead citrate for 5 minutes before observation by employing a JEM 1400 plus transmission electron microscope at 100 kV.

Intracellular and exosome sodium detection. 2×10^6 toad UB epithelial cells enriched by centrifugation were resuspended with Ringer’s solution (N-Methyl-D-glucamine was used instead of NaCl) after digestion and washing 3 times. MDCC cells were cultured to 90% on 100-mm cell culture dishes, and three cell culture dishes in each group were used in this experiment. Toad UB epithelial cells and MDCC cells were cultured for 3 hours with and without 50 nM or 10 nM βγ-CAT, respectively. The cells were collected, and lysed with deionized water. Sodium concentrations in MDCC exosomes were measured in isotonic or hypertonic assays using forty or twenty cell culture dishes in each group, respectively. MDCC exosomes were isolated as described in “Isolation of exosomes”. The samples were frozen in liquid nitrogen and then slowly melted at room temperature, and repeated twice. Ultrasonic cell crushing (power 200 W, work every 10 seconds for 3 seconds, 5-40 cycles) was used to release the sample until the liquid was transparent. Impurities in samples were removed by gradient centrifugation (2000 x g for 30 minutes; 10,000 x g for 1 hour). After the sample was filtered with a 0.22 μm filter, the concentration of sodium ions in the samples was determined by iCAP6300 inductively coupled plasma optical emission spectrometer (Thermo Scientific, Waltham, United States of America).

Statistical analysis. Animal survival data were analyzed by the Gehan-Breslow-Wilcoxon test. All other data were analyzed using the standard unpaired t-test. Differences with P values < 0.05 were considered statistically significant. All statistical analyses were conducted using GraphPad Prism 8 software.
Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. The sources for all data supporting the results of this study are cited in the main text, in Supplementary figures, and in Supplementary table 1. Uncropped and unedited blot/gel images as Supplementary fig. 5 at the Supplementary information. The datasets generated during and/or analysed during the current study are available in the Dryad Data repository (https://doi.org/10.5061/dryad.fp2mg126). All clipart components in Fig. 6 are sourced from PowerPoint 2019 and Adobe illustrator CC 2019.

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References

1. Bentley, P. J. Adaptations of amphibia to arid environments. Science 152, 619–623 (1966). 2. Shoemaker, V. & Nagy, K. A. Osmoregulation in amphibians and reptiles. Am. Zool. 19, 449–471 (1979). 3. Hillyard, S. D. Behavioral, molecular and integrative mechanisms of amphibian osmoregulation. J. Exp. Zool. 283, 662–674 (1999). 4. Ogushi, Y. et al. The water-absorption region of ventral skin of several semi-arid and semiterrestrial amphibians identified by aquaporins. Arch. Histol. Cytol. 74, 115–126 (2011). 5. Suzuki, M., Hasegawa, T., Ogushi, Y. & Tanaka, S. Amphibian aquaporins and their functional roles in water transport. Comp. Biochem. Physiol. A. Mol. Integr. Physiol. 152, 72–81 (2007). 6. Kerr, M. C. & Teasdale, R. D. Defining macropinocytosis. Traffic 10, 364–371 (2009). 7. Swanson, J. A. & Watts, C. Macropinocytosis. Trends Cell Biol. 5, 424–428 (1995). 8. Ramirez, C., Hauser, A. D., Vucic, E. A. & Bar-Sagi, D. Plasma membrane V-ATPase controls oncogenic RAS-induced macropinocytosis. Nature 576, 477–481 (2019). 9. Breton, M. et al. Lysosome signaling controls the migration of dendritic cells. Sci. Immunol. 2, eaax9573 (2017). 10. Palm, W. & Thompson, C. B. Nutrient acquisition strategies of mammalian cells. Nature 546, 234–242 (2017). 11. Palm, W. Metabolic functions of macropinocytosis. Philos. Trans. R. Soc. B Biol. Sci. 374, 20180285 (2019). 12. Swanson, J. A. & King, J. S. The breadth of macropinocytosis research. Philos. Trans. R. Soc. B Biol. Sci. 374, 20180146 (2019). 13. Parker, M. W. et al. Structure of the β2γ subunit complex from an IgG Fc receptor modulates cellular responses. J. Immunol. 191, 13609–13625 (2013). 14. Gao, Q. et al. Identification of novel macropinocytosis inhibitors using a rational screen of Food and Drug Administration-approved drugs. Br. J. Pharmacol. 175, 3640–3655 (2018). 15. Li, H. et al. Identification of novel macropinocytosis inhibitors using a rational screen of Food and Drug Administration-approved drugs. Br. J. Pharmacol. 175, 3640–3655 (2018). 16. Hays, R. M. & Leaf, A. Studies on the movement of water through the isolated toad bladder and its modification by vasopressin. J. Gen. Physiol. 45, 905–919 (1962). 17. Hasegawa, T., Suzuki, M. & Tanaka, S. Immunocytochemical studies on translocation of phosphorylated aquaporin-h2 protein in granular cells of the frog urinary bladder before and after stimulation with vasotocin. Cell Tissue Res. 322, 407–415 (2005). 18. Davis, C. W. & Finn, A. L. Interactions of sodium transport, cell volume, and calcium in frog urinary bladder. J. Gen. Physiol. 89, 687–702 (1987). 19. van Niel, G., D’Angelo, G. & Raposo, G. Shedding light on the cell biology of extracellular vesicles. Nat. Rev. Mol. Cell Biol. 19, 213–228 (2018). 20. Gao, Q. et al. β2γ-CAT, a non-lens betagamma-crystallin and trefoil factor complex, induces calcium-dependent platelet aggregation. Thromb. Haemost. 105, 846–854 (2011). 21. Zhang, Y. X., Lai, R., Lee, W. H. & Zhang, Y. Frog albumin is expressed in skin and characterized as a novel potent trypsin inhibitor. Protein Sci. 14, 2469–2477 (2005). 22. Zhao, F. et al. Comprehensive transcriptome profiling and functional analysis of the frog (Bombina maxima) immune system. DNA Res. 21, 1–13 (2014). 23. Freeman, S. A. et al. Lipid-gated monovalent ion fluxes regulate endocytic traffic and support immune surveillance. Science 367, 301–305 (2020). 24. King, J. S. & Smythe, E. Water loss regulates cell and vesicle volume. Science 367, 246–247 (2020). 25. Russell, A. E. et al. Biological membranes in EV biogenesis, stability, uptake, and cargo transfer: an ISEV position paper arising from the ISEV membranes and cargo transfer: an ISEV position paper. J. Extracell. Vesicles 3, 13625 (2014). 26. Liu, L. et al. An aerolysin-like pore-forming protein complex targets viral envelope to inactivate Herpes Simplex Virus Type 1. J. Immunol. 207, 888–891 (2021). 27. Larsen, E. H. Dual skin functions in amphibian osmoregulation. Comp. Biochem. Physiol. A. Mol. Integr. Physiol. 253, 110869 (2021). 28. Takata, K. Aquaporins: water channel proteins of the cell membrane. Prog. Histochem. Cytochem. 39, 1–83 (2004). 29. de Baer, A. & Lanuzucchia, A. The role of aquaporins in dendritic cell macrophagocytosis. J. Exp. Med. 191, 743–748 (2000). 30. Nonnenmacher, M. & Weber, T. Adeno-associated virus 2 infection requires endocytosis through the CLIC/GEEC pathway. Cell Host Microbe 10, 563–576 (2011). 31. Lim, J. P. & Glesson, P. A. Macropinocytosis: an endocytic pathway for engulfing large guls. Immunol. Cell Biol. 89, 836–843 (2011). 32. Challa, D. K. et al. Neutonal Fc receptor expression in macrophages is indispensable for IgG homeostasis. mAbs 11, 848–860 (2019). 33. Araki, N., Johnson, M. T. & Swanson, J. A. A role for phosphoinositide 3-kinase in the completion of macropinocytosis and phagocytosis by macrophages. J. Cell Biol. 135, 1249–1260 (1996). 34. Lin, H. P. et al. Identification of novel macropinocytosis inhibitors using a rational screen of Food and Drug Administration-approved drugs. Br. J. Pharmacol. 175, 3640–3655 (2018). 35. Gao, Q. et al. β2γ-CAT, a non-lens betagamma-crystallin and trefoil factor complex, induces calcium-dependent platelet aggregation. Thromb. Haemost. 105, 846–854 (2011). 36. Zhang, Y. X., Lai, R., Lee, W. H. & Zhang, Y. Frog albumin is expressed in skin and characterized as a novel potent trypsin inhibitor. Protein Sci. 14, 2469–2477 (2005). 37. Zhao, F. et al. Comprehensive transcriptome profiling and functional analysis of the frog (Bombina maxima) immune system. DNA Res. 21, 1–13 (2014). 38. Freeman, S. A. et al. Lipid-gated monovalent ion fluxes regulate endocytic traffic and support immune surveillance. Science 367, 301–305 (2020). 39. King, J. S. & Smythe, E. Water loss regulates cell and vesicle volume. Science 367, 246–247 (2020). 40. Russell, A. E. et al. Biological membranes in EV biogenesis, stability, uptake, and cargo transfer: an ISEV position paper arising from the ISEV membranes and cargo transfer: an ISEV position paper. J. Extracell. Vesicles 3, 13625 (2014). 41. Holrich, V., Mühler, L., Arvestad, L. & Sonnhammer, E. L. L. Assessment of protein distance measures and tree-building methods for phylogenetic tree reconstruction. Mol. Biol. Evol. 22, 2257–2264 (2005).
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Author contributions
These authors contributed equally to this work: Z.Z., and Z.H.S. Correspondence to Y.Z. Y.Z., Z.Z., and Z.H.S conceived of and conceptualized the study; Z.Z., Z.H.S., and C.J.Y did the experiments, Z.Z., Y.Z., Z.H.S, and C.J.Y analyzed and interpreted the data; Z.Z., Y.Z., Z.H.S wrote the manuscript; Y.Z., Z.Z., Z.H.S and C.J.Y critically revised the manuscript for important intellectual content.

Competing interests
The authors declare no competing interests.

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