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The Activation Pathway of the Volume-Sensitive Organic Osmolyte Channel in *Xenopus laevis* Oocytes Expressing Skate Anion Exchanger 1 (AE1)

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Abstract. When swollen, skate red blood cells increase permeability and allow efflux of a number of solutes, including taurine. Hypoosmosis-induced taurine permeability appears to involve the red cell anion exchanger. However, three isoforms have been cloned from these cells. Therefore, to determine the ability of the individual isoform skate anion exchanger 1 (skAE1) to mediate hypoosmosis-induced taurine permeability as well as associated regulatory events, skAE1 was expressed in *Xenopus* oocytes. This study focused on investigating the role of tyrosine kinases and lipid rafts in the regulation of the channel. The results showed that tyrosine kinase inhibitors and lipid raft-disrupting agents inhibited the volume-sensitive organic osmolyte channel while protein tyrosine phosphatase inhibitors activated the channel in oocytes expressing skAE1. To study the role of lipid rafts in the activation of the volume-sensitive organic osmolyte channel, the cellular localization of skAE1 was investigated. Also, the role of tyrosine kinases was investigated by examining the tyrosine phosphorylation state of skAE1. Hypoosmotic stress induced mobilization of skAE1 into light membranes and the cell surface as well as tyrosine phosphorylation of skAE1. These events are involved in the activation of the volume-sensitive organic osmolyte channel in *Xenopus* oocytes expressing skAE1.

Key words: Organic osmolyte channel — Skate AE1 — *Xenopus laevis*

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Introduction

Cell volume regulation is essential for all cells. Upon cell swelling due to hypotonic conditions, efflux of osmotically active solutes (osmolytes) increases with a subsequent loss of water, resulting in the restoration of cell volume, termed the *regulatory volume decrease* (RVD) [11, 12, 16, 20, 21, 30]. A wide variety of cells use organic osmolytes to accomplish RVD. Red blood cells of both bony teleost (trout) and cartilaginous (skate) fish use the nonmetabolized β-amino acid taurine as a major osmolyte in volume regulation. The RVD of skate erythrocytes involves efflux of taurine through a volume-sensitive organic osmolyte permeability [3–5, 8, 17, 28]. In skate erythrocytes, volume-stimulated taurine efflux occurs through a bidirectional, sodium-independent, nonselective channel with broad specificity. A variety of solutes may pass through this hypoosmosis-induced channel/permeability pathway, including amino acids, sugar alcohols, and tertiary amines [7]. The hypoosmosis-induced permeability was strongly suggested to be a channel allowing some solutes under a hydrated diameter of 6 Å to pass [7]. The permeation of a number of solutes of varying solute classes (taurine as well as other amino acids, betaine and related solutes, as well as sorbitol) suggests that this is a channel- and not a carrier-mediated transport. The permeability induced by hypoosmotic medium in skate erythrocytes has solute characteristics different from the volume-stimulated organic osmolyte channel/volume-stimulated organic anion channel permeability which occurs in a variety of other cells. In many, but not all cells, cell volume increases induced by volume expansion in hypoosmotic medium increase efflux of not only taurine, as well as sorbitol and betaine, but also nonorganic anions such as Cl^- [29, 37]. The precise nature of the protein(s) which mediates the volume-stimulated solute efflux is not

always known, and a number of candidates have been identified including members of the ClC channel family, the anion channel pICln, and members of the anion exchanger family (as appears to occur in both skate and trout erythrocytes). Even within the erythrocytes, the permeabilities that the anion exchangers confer may differ. Trout anion exchanger 1 (AE1) expressed in *Xenopus* oocytes confers not only taurine permeability but also K^+ permeability, whereas skate AE1 (skAE1) allows taurine to pass but not K^+ [14]. Therefore, although these exchangers do share significant homology, sufficient differences exist to allow differential solutes to pass. It should be noted that *Xenopus* oocytes do have endogenous volume-stimulated anion efflux, but this has permeability properties different from the permeability conferred by expression of trout AE1 or skAE1 [13]. In addition, the endogenous ability for volume-stimulated solute efflux to occur is generally lost within 1-2 days after isolation, a time when the protein from the injected RNA is beginning to be expressed [13].

Biochemical and pharmacological data suggest that the skAE1 protein plays a key role in the activation of this volume-sensitive organic osmolyte channel [7, 28, 30]. In skate erythrocytes, regulation of the volume-sensitive organic osmolyte channel may involve multiple events, including modulation of tyrosine kinases and tyrosine phosphorylation of skAE1 [9, 23, 26, 27, 30], association of skAE1 with regulatory proteins (ankyrin and band 4.1), insertion of skAE1 into the surface cell membrane from an intracellular pool (both in buoyant regions of non-ionic detergent-insoluble membrane material), and oligomerization of skAE1 into tetramers [22, 24, 25, 27, 30]. Recently, mRNA encoding three anion exchanger isoforms, skAE1, skAE2, and skAE3, has been cloned from skate red blood cells [6]. The skAE1 protein was shown to be present in skate erythrocytes, but it is not known whether the other two isoforms, skAE2 and skAE3, are also expressed at the protein level. The presence of three AE isoforms raises the question as to their function in the erythrocyte. Also, the question remains regarding the identification of the AE isoform involved in activating the volume-sensitive organic osmolyte channel in skate erythrocytes.

The *Xenopus laevis* oocyte system provides an opportunity to express each of the skAEs and study their regulation, isolated from activities of the other two isoforms. skAE1 and skAE2 as well as trout AE1 have been expressed in these oocytes and demonstrated to possess anion exchange activity [6, 13, 14]. A recent study revealed that a volume-sensitive organic osmolyte channel could be induced by expressing skAE1 in *Xenopus* oocytes and that the channel opening/activity was stimulated by hypoosmotic stress [14, 15]. In the present studies, we used the *Xenopus* oocyte expression system to investigate the importance of the phosphorylation state of skAE1, mobilization of skAE1 into light membranes, and surface expression of skAE1 in order to elucidate the effects of hypoosmotic stress on these events and the possible role of these events in the activation pathway of the volume-sensitive organic osmolyte channel in skAE1-expressing oocytes. We demonstrate that the sensitivity of the channel to pharmacological inhibitors in the isolated system is similar to that in the red blood cell.

Materials and Methods

Expression of skAE1 in *X. laevis* Oocytes

Skate (*Raja erinacea*) erythrocyte anion exchanger (skAE1) cRNA was prepared from skAE1 cDNA cloned into the vector pGEM-T easy, linearized with ApaI and transcribed using the Ambion mMessage mMachine kit (Austin, TX). The cRNA was resuspended in RNAse-free water with RNAsin and stored at −80°C. An aliquot was analyzed by agarose-formaldehyde gel electrophoresis prior to each experiment.

Oocytes were surgically removed from adult female *X. laevis* anesthetized with 0.3% (wt/vol) 3-aminobenzoic acid ethyl ester (MS-222). Oocytes were separated and defolliculated by treatment with 2 mg/ml collagenase and 1 mg/ml trypsin inhibitor in oocyte medium ND109 (109 mM NaCl, 2.0 mM KCl, 1.8 mM $CaCl_2$, 1.0 $MgCl_2$, 5.0 mM 4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid [HEPES, pH 7.5], 235 mOsm, filtered). Defolliculated oocytes were washed with ND109, and stage V-VI oocytes were selected and maintained in ND109 supplemented with penicillin (10 U/ml) and streptomycin (10 μg/ml) at 18°C overnight. Oocytes were injected with skAE1 cRNA (50 nl at a concentration of 6 ng/50 nl) or water as a control for injection and volume effects.

Taurine Uptake in skAE1-Expressing Oocytes

Xenopus oocytes injected with skAE1 RNA were assayed for volume-stimulated organic osmolyte channel induction by measuring radiolabeled taurine uptake. After 72 h postinjection incubation at 18°C, 10–12 oocytes injected with skAE1 were first washed in ice-cold ND109 two times, then transferred to wells containing 0.4 ml ND109 or ND96 (NaCl reduced to 96 mM) for isoosmotic and hypoosmotic conditions with 0.1 mM taurine and 2 μCi/ml [^{3}H]-taurine. Ten microliters of the incubation medium from each group was taken at $t = 0$ to determine specific activities. After incubating in appropriate media for varying time, oocytes were washed three times in ice-cold uptake medium and quickly transferred to scintillation vials. Twenty microliters of 20% sodium dodecyl sulfate (SDS) was added to all the tubes, and then liquid scintillation fluid was added to all vials at least 3 h later. The vials were capped, vortexed, placed in a liquid scintillation spectrometer, and analyzed for radioactivity. Uptakes are presented as picomoles of taurine per hour per oocyte. In some experiments, taurine transport was measured in hypoosmotically stressed skAE1-expressing oocytes in the presence of various kinase and phosphatase inhibitors (tyrosine kinase inhibitors piceatannol and tyrphostin 23, mitogen-activated protein kinase inhibitor PD98059, protein kinase A inhibitor H-89, protein kinase C inhibitor bisindolylmaleimide, phosphoinositide-3 kinase inhibitor LY294002, protein tyrosine phosphatase inhibitor pervanadate). To test the effects of piceatannol, oocytes were preincubated in piceatannol for 30 min before taurine uptake measurements.

Vanadate was oxidized to pervanadate by hydrogen peroxide immediately before use by the reaction of hydrogen peroxide (3 mM) with a stock solution of 1 mM Na orthovandate and used at a 1:10 dilution, resulting in a concentration of pervanadate of 0.1 mM.

Western Blots

From each group, 20–40 oocytes were combined and homogenized in 5 ml lysis buffer (50 mM Tris [pH 7.4], 66 mM ethylenediaminetetraacetic acid [EDTA], 1% vol/vol Triton X-100, 0.4% wt/vol deoxycholic acid, with the complete protease inhibitor cocktail; Roche Molecular Biochemicals, Indianapolis, IN). Samples were homogenized with a tight-fitting Teflon pestle homogenizer, then centrifuged at 3,000 × *g* for 5 min at 4°C. Pellets were washed in 500 μl lysis solution, homogenized again, and centrifuged at 3,000 *g* for 5 min at 4°C. The process was repeated a third time to clear the supernatant. The pellets were homogenized again in 500 μl lysis buffer and centrifuged at 15,000 × *g* for 30 min at 4°C to obtain a crude membrane fraction. Pellets were resuspended in immunoprecipitation (IP) buffer (150 mM NaCl, 10 mM Tris-HCl [pH 7.4], 2 mM EDTA, 1% vol/vol Triton X-100, 0.5% wt/vol deoxycholate, 0.1% wt/vol SDS, 0.1 mM sodium vanadate, 1 mM sodium fluoride). Protein concentrations were determined by a bicinchoninic acid procedure. Twenty micrograms of protein from the membrane preparation were loaded on 10% SDS polyacrylamide gels. The samples were resolved and transferred to polyvinylene difluoride membranes. The blots were blocked in Tween Tris-buffered saline (T-TBS; 140 mM NaCl, 5 mM KCl, 10 mM Tris [pH 7.4], with 0.05% vol/vol Tween-20) plus bovine serum albumin (3% wt/vol) for 2 h, then incubated overnight with rabbit polyclonal anti-skAE1 or mouse monoclonal antiphosphotyrosine (Upstate Biotechnology, Charlottesville, VA; clone 4G10). The blots were washed five times in T-TBS, incubated with the appropriate peroxidase-conjugated secondary antibodies, and washed four more times with T-TBS and once in TBS. The blots were then developed using an enhanced chemiluminescent system.

Immunoprecipitation

Twenty micrograms of protein from the membrane preparation prepared above were incubated with Pansorbin (heat-killed *Staphlococcus aureus*) to preclear, and then agarose beads which had been conjugated to the rabbit polyclonal anti-skAE1 antibody using the Pierce (Rockford, IL) Seize-X kit were added and the samples incubated overnight. The samples were gently centrifuged and the supernatant was removed. Beads were washed four times in IP buffer and immunoprecipitated proteins eluted in immunoglobulin G elution buffer (Pierce). Half of the eluted protein was added to 3× Laemmli stop solution, heated at 65°C, and analyzed by Western blot, as described above. Half of the eluted protein was added to strepavidin agarose (Pierce) and biotinylated proteins isolated by incubation for 2 h. Beads were centrifuged and washed with IP buffer three times, and biotinylated proteins were eluted with 1× Laemmli stop solution, heated at 65°C for 10 min, and analyzed by Western blotting.

Mobilization of skAE1 into Light Membranes

A detergent-free procedure was used for the extraction of light membranes that have characteristics and properties similar to detergent-resistant membranes [19]. Light membranes were isolated from oocyte membranes as described by Luria et al. [19]. Twenty to fifty oocytes from each group were lysed by homogenization in ice-cold 2× TNE buffer (10 mM Tris, 150 mM NaCl, 5 mM EDTA [pH 7.4], with complete protease inhibitor cocktail, 1 mM benzamidine, and 1 mM sodium vanadate) using a Teflon homogenizer. The samples were centrifuged at 3,000 × *g* for 10 min; the supernatant was removed and the pellets were resuspended in TNE buffer. This process was repeated three times, and the membranes were resuspended in 1 ml 2× TNE buffer. The oocyte lysates were then sonicated, and an equal volume of 80% sucrose was added. Three layers, each of 3 ml, were layered on top of 35%, 22.5%, and 10% wt/vol sucrose in 2× TNE. The gradient was centrifuged for 3 h at 100,000 × *g* at 4°C, and the fractions were collected from the interfaces and the bottom. The light fraction from membranes of *Xenopus* oocytes expressing skAE1 or control oocytes was isolated at the interface of the 10–22.5% layers and probed for skAE1 and tyrosine-phosphorylated skAE1 localized in the light membranes. skAE1 was immunoprecipitated from the light membranes as described above; in some cases, biotinylated skAE1 was subsequently isolated from the immunoprecipitated skAE1. The light membrane fraction was also analyzed directly (without immunoprecipitation) for markers of membrane domains termed *lipid rafts*, caveolin-1 (Upstate Biotechnology), flotillin-2 (Santa Cruz Biotechnology, Santa Cruz, CA), or the heavy chain of clathrin (BD Transduction Labs, San Diego, CA) by Western blotting as described above. Cholesterol was also measured in these membranes by extraction into chloroform using chloroform:methanol:acetic acid (200:100:4) and drying down the chloroform layer. Cholesterol in this extract was measured using the Amplex Red assay (Molecular Probes-Invitrogen, Eugene, OR), and cholesterol content was calculated per protein in the oocyte membranes.

Biotinylation

Oocytes were incubated in ND109 or ND96 (isoosmotic and hypoosmotic conditions) in the presence or absence of inhibitors as designated. The medium was removed and replaced with ice-cold medium of identical osmolarity. A 2 mg/ml solution of the cell-impermeant form of biotin, sulfo-NHS-biotin (Pierce), in ND96 was prepared and chilled at 4°C. An equal volume of chilled biotin was added to cells to obtain a final concentration of 1 mg/ml biotin. The cells were then incubated in the cold for 1 h. The reaction was stopped by adding 1/100 volume of 1 M Tris, pH 7.4. The cells were washed two times with appropriate osmolarity ND medium with 10 mM Tris added and spun down, and the supernatant was removed. The total cell or light membranes from each sample were isolated as described above. Fifty microliters of strepavidin-agarose were added, and the samples were rotated in the cold room for 2 h. Beads were gently pelleted and washed, and biotinylated protein was eluted. The supernatant was saved and analyzed by Western blot as described above.

Statistics

Three to five batches of oocytes (from different frogs) were used for each flux experiment, a total of 30–36 oocytes/experiment. Significance was determined using analysis of variance with a Bonferroni correction or paired Student's *t*-test using Instat software for Macintosh (GraphPad, San Diego, CA).

Results

Effect of Tyrosine Kinase and Phosphatase Inhibitors

Many uptake studies in *Xenopus* oocytes have used the medium ND96, whose composition results in a

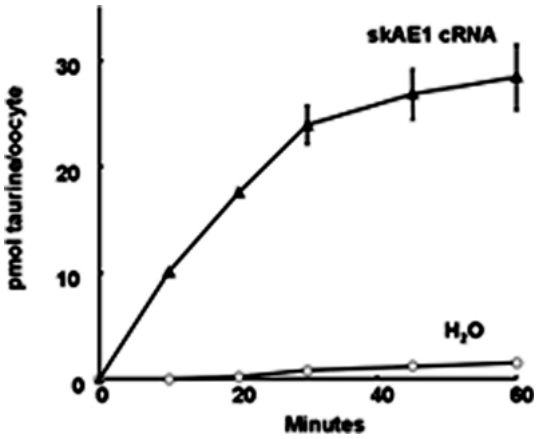

Fig. 1. Time course of taurine uptake in *Xenopus* oocytes expressing skAE1. Oocytes were injected with either skAE1 cRNA or water and incubated for 3 days in ND109 (235 mosmol/liter). On the third day, both groups were transferred to ND96 (210 mosmol/liter) and taurine uptake was measured as described in Materials and Methods. Values are means ± standard error for three separate experiments. At some time points, the standard error bar is too small to be shown.

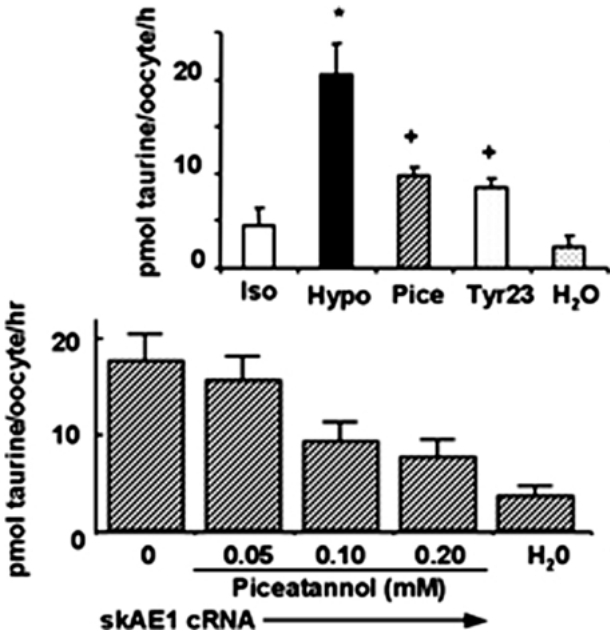

Fig. 2. Effects of tyrosine kinase inhibitors on volume-stimulated taurine transport. (*A*) skAE1 was expressed in *Xenopus* oocytes and incubated in isoosmotic modified Ringer's (Iso, 235 mOsm), hypoosmotic ND96 (Hypo, 210 mOsm), or hypoosmotic ND96 in the presence of the tyrosine kinase inhibitors piceatannol (Pice, 0.1 mM) and tyrphostin A23 (Tyr23, 0.1 mM). Control, water-injected oocytes were incubated in hypoosmotic ND96 (H_2O). Values are expressed as means ± standard error, $^{+}P < 0.01$ hypoosmotic vs. hypoosmotic and inhibitor, $*P < 0.01$ hypoosmotic vs. control and isoosmotic by analysis of variance. (*B*) Concentration dependence of piceatannol inhibition of hypoosmosis-induced taurine uptake. Oocytes were injected with skAE1 cRNA (all except water) or water (H_2O) and kept in ND109 for 3 days, when all oocytes were transferred to ND96 and taurine uptake was measured under hypoosmotic conditions for all as described in Materials and Methods. When appropriate, oocytes were treated with varying concentrations of piceatannol for 60 min before uptake. Values are means ± standard error for three separate experiments.

medium osmolarity of approximately 210 mosmoles/liter. When oocytes are injected with skAE1 cRNA and incubated in this medium, anion exchange as well as increased basal permeability to taurine are observed with little change upon hypotonic exposure [14, 15]. Increasing osmolarity to 235 mM (the osmolarity of *Xenopus* extracellular fluid (ECF) [15]) with the addition of 15 mM NaCl decreases basal taurine permeability and results in the ability to observe hypoosmosis-induced increased taurine permeability [15], allowing investigation of events regulating skAE1 as a taurine channel. In the present studies, to determine a time point where uptakes after varying treatments could be compared, a time course was performed. Hypoosmosis (ND96)-stimulated uptake reached a plateau near 30 min. Therefore, this time point was chosen for all subsequent fluxes (Fig. 1).

Protein kinases and phosphatases have been shown to be involved in the regulation of volume-activated osmolyte channels in many cell types, including erythrocytes of a number of species (including skate) as well as endothelial and epithelial cells as limited examples [1, 2, 9, 23, 26, 30]. In order to ascertain whether tyrosine kinase or phosphatase activities are involved in the activation pathway of the volume-sensitive organic osmolyte channel induced by skAE1 expression in *Xenopus* oocytes, a variety of inhibitors were used. Taurine transport was measured in hypoosmotically stressed skAE1-expressing oocytes treated with the following inhibitors: (1) tyrphostin 23, a tyrosine kinase inhibitor which inhibits a variety of tyrosine kinases, and (2) piceatannol, which inhibits p^{72}syk at low concentrations and p^{56}lyn and src-family tyrosine kinases at higher concentrations and has been demonstrated to inhibit hypoosmosis-induced taurine efflux in skate red blood cells. Both piceatannol and tyrphostin 23 inhibited hypoosmotically stimulated taurine uptake induced by skAE1 expression in *Xenopus* oocytes significantly, by 53% and 59%, respectively (Fig. 2). The inactive tyrphostin analog A46 did not inhibit taurine transport (*data not shown*). The effect of piceatannol was concentration-dependent (Fig. 2) and is in agreement with its effects on inhibition of the tyrosine kinase p^{72}syk [26]. These data support a role for tyrosine phosphorylation, possibly p^{72}syk, in the hypoosmosis-induced activation of skAE1 in oocytes, although we cannot exclude involvement of other tyrosine kinases.

To determine whether other kinases, including certain serine/threonine kinases, might be involved in hypoosmosis-induced taurine permeability, the fol-

lowing were examined: (1) the protein kinase C inhibitor bisindolylmaleimide (BIM), (2) the protein kinase A inhibitor H-89, (3) the mitogen-activated protein kinase inhibitor PD98059, and (4) the phosphoinositide-3 kinase inhibitor LY294002. BIM (100 μm) caused a small, not significant inhibition of taurine uptake (32.4 ± 4.8 without and 28 ± 3.5 pmol taurine/oocyte/h [n = 30] with 100 μM BIM). LY294002 (100 μM) inhibited taurine transport by 30% ($P < 0.05$, n = 24), 22.4 ± 1.8 (n = 34) pmol taurine/oocyte/h compared to hypoosmosis-stimulated skAE1 oocytes in the absence of the inhibitor, 33.5 ± 4.1 (n = 36) pmol taurine/oocyte/h. H-89 and PD 98059 (100 μM each) had no effect on taurine uptake in hypoosmotically stressed skAE1 oocytes (38 ± 4.5 pmol taurine/oocyte/h [n = 30] hypoosmotic control vs. 37 ± 7.8 [n = 36] and 39 ± 8 [n = 36] with H-89 and PD98059, respectively). These results indicate that while phosphoinositide-3 kinase may play a small role in the activation of the volume-sensitive organic osmolyte channel in skAE1 oocytes, protein tyrosine kinases and phosphatases are the major signaling pathways involved in the regulation of the channel in these cells.

Further evidence for the involvement of tyrosine phosphorylation was obtained using the protein tyrosine phosphatase inhibitor pervanadate. Since the oxidized form of vanadate, pervanadate, is the most potent phosphatase inhibitor, vanadate was reacted with hydrogen peroxide before use at a molar ratio of 1 vanadate to 3 hydrogen peroxide. Pervanadate treatment (0.1 mM for 10 min) increased taurine permeability under isoosmotic conditions and was not additive when used in hypoosmotic medium (Fig. 3). Since hydrogen peroxide itself has been demonstrated to affect certain permeabilities involved in solute loss in RVD [34, 35], we tested the effects of adding hydrogen peroxide alone. No significant effects of hydrogen peroxide on basal or volume-stimulated organic osmolyte channel in skAE1 oocytes were observed. Taurine transport measured in skAE1-expressing oocytes incubated in isoosmotic, isoosmotic plus hydrogen peroxide, hypoosmotic, and hypoosmotic plus hydrogen peroxide media were 6.1 ± 2.7 (n = 35), 7.8 ± 2.3 (n = 34), 26.7 ± 3.9 (n = 36), and 29.8 ± 4.6 (n = 32) pmol taurine/oocyte/h, respectively. Therefore, the effects of pervanadate were not due to the additive effect of hydrogen peroxide.

To confirm that pervanadate increased skAE1 tyrosine phosphorylation, total oocyte membranes were isolated, skAE1-immunoprecipitated, and probed by Western blotting for skAE1 and phosphotyrosine. Pervanadate treatment increased skAE1 tyrosine phosphorylation analyzed in the total membranes, and its effect was similar to the increase observed with hypoosmotic exposure (third panel on left, Fig. 4). No differences were noted in the total

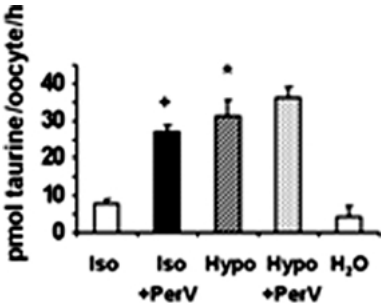

Fig. 3. Effects of protein tyrosine phosphatase inhibitor pervanadate on volume-stimulated taurine transport. skAE1 was expressed in *Xenopus* oocytes and incubated in isoosmotic ND109 (Iso, 235 mOsm), isoosmotic ND109 with pervanadate (0.1 mM, Iso + PerV), hypoosmotic ND96 (Hypo, 210 mOsm), or hypoosmotic ND96 with pervanadate (0.1 mM, Hypo + PerV). Control, water-injected oocytes were incubated in hypoosmotic ND96. Values are expressed as means ± standard error, $^{+}P < 0.01$ isoosmotic vs. isoosmotic and pervanadate and $*P < 0.01$ hypoosmotic vs. isoosmotic by analysis of variance.

amount of skAE1 in total membranes from oocytes in isoosmotic or hypoosmotic conditions with or without pervanadate (top panel on left, Fig. 4). There was no further increase in tyrosine phosphorylation when pervanadate was added to oocytes incubated in hypoosmotic medium. The pervanadate-induced increase in skAE1 tyrosine phosphorylation was observed not only in total membranes (isolated using the nonionic detergent Triton X-100) but also in surface-expressed skAE1 in the total membranes (biotinyl skAE1; fourth panel on left, Fig. 4). Additionally, pervanadate stimulated tyrosine phosphorylation of skAE1 in oocyte light membranes (third panel on right, Fig. 4), an effect also observed for skAE1 in light membranes in surface plasma membranes as demonstrated by biotinylated skAE1 in light membranes (fourth panel on right, Fig. 4). It is also noted that pervanadate treatment increased skAE1 in the light membranes (first panel on right, Fig. 4) and in surface-expressed skAE1 in the light membranes (second panel on right, Fig. 4). Potential mechanisms for this effect will be presented in the Discussion but suggest that the state of tyrosine phosphorylation of skAE1 may influence its distribution in membrane microdomains as well as localization in the cell.

To confirm that these light membranes possessed some protein markers similar to buoyant lipid raft domains of plasma membranes, the expression of markers flotillin-2 and caveolin-1, both markers of buoyant lipid raft regions, as well as of the heavy chain of clathrin, which is one of the clathrin subunits found in clathrin-coated pits, was determined in light membranes. Caveolin-1 as well as flotillin-2 are highly enriched in light membranes compared with the total membranes (Fig. 5). What is termed *total oocyte membranes* is isolated using 1% vol/vol Triton

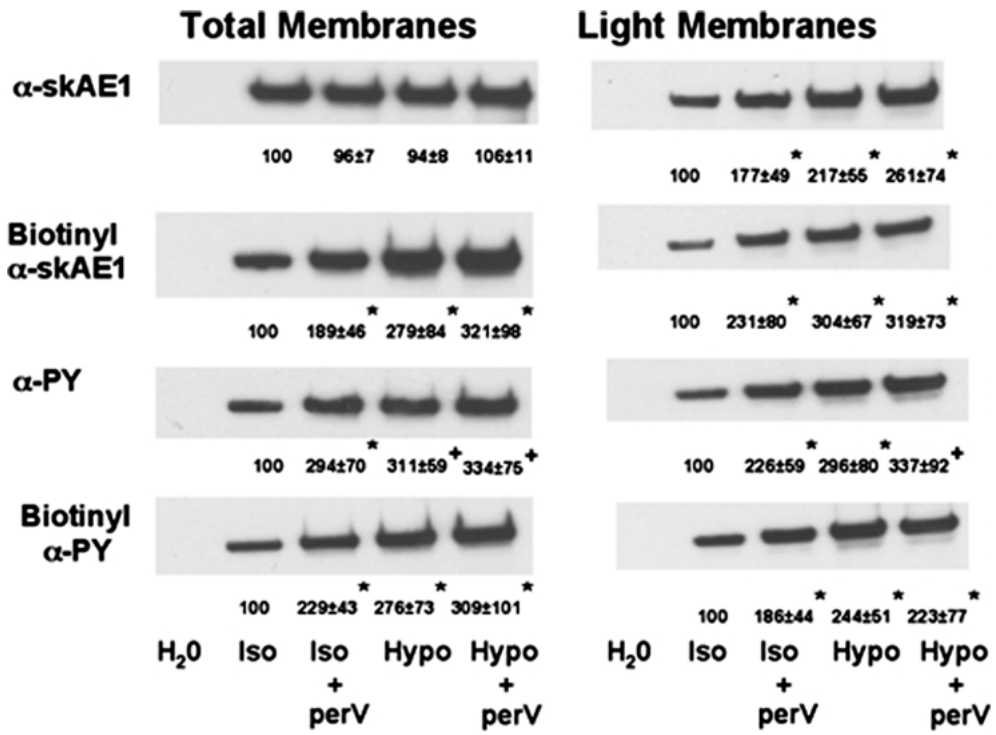

Fig. 4. Effect of pervanadate on skAE1 localization and tyrosine phosphorylation following hypoosmotic exposure. Water- or skAE1 cRNA-injected oocytes were kept in ND109 for 3 days and then, when appropriate, treated with 0.1 mM pervanadate for 60 min prior to transfer to hypoosmotic ND96 for 60 min. Oocytes were then biotinylated as described in Materials and Methods at 4°C for 30 min. skAE1 as well as its state of tyrosine phosphorylation (PY) were analyzed in both total membranes (*all panels on left*) and light membranes isolated on a discontinuous sucrose gradient (*all panels on right*). skAE1 was immunoprecipitated and analyzed for skAE1 (*top panels on right and left*) or phosphotyrosine (*third panels down on right and left*) using anti-skAE1 or anti-phosphotyrosine antibodies and Western blotting. A portion of the immunoprecipitation was further incubated with streptavidin agarose; therefore, only skAE1 at the surface was isolated. This surface-expressed skAE1 was also analyzed for the abundance of skAE1 and its level of tyrosine phosphorylation. Images are representative of four separate experiments. In all cases, isoosmosis was set to 100 and densitometry was performed, with increases based on fold increases over this value for each condition. $^{*}P < 0.05$, $^{+}P < 0.01$, iso + perV vs, iso vs, hypo and hypo + perV vs. hypo were compared, all comparisons by paired Student's *t*-test.

X-100, a nonionic detergent which permits easier membrane isolation in oocytes due to the large amount of yolk protein but may not result in the isolation of all membranes. The light membranes contained little heavy chain of clathrin, suggesting differences in the density of clathrin-containing regions and areas with caveolin-1 and/or flottilin-2, where the membrane may have more cholesterol and be more buoyant. The results suggest that in the oocyte, as in the intact skate erythrocyte, skAE1 is directed to lipid rafts and this may be an important facet of its regulation. The importance of the clathrin-containing regions does not appear to play a role in expanded volume-induced increased skAE1 surface expression, but whether clathrin-containing regions might play a role in other facets of skAE1 regulation, e.g., endocytosis after surface expression, is unknown and important to document in the future.

To help establish the importance of cholesterol-rich membrane domains in oocyte regulation of taurine permeability conferred by skAE1, the cholesterol-depleting agent methyl-β-cyclodextrin (MBCD) was used. In a concentration-dependent manner, MBCD pretreatment inhibited the hypoosmosis-induced increase in taurine uptake in oocytes (Fig. 6). Under isoosmotic conditions, cyclodextrin had no effect on taurine uptake (*data not shown*). To confirm that cyclodextrin pretreatment did indeed disrupt oocyte light membranes, the cholesterol and protein content of light membranes was determined. Light membranes from water-injected oocytes or those from skAE1 cRNA-injected oocytes in isoosmotic or hypoosmotic conditions had similar protein and cholesterol contents. When expressed on a per egg basis, for water-injected oocytes the values were 0.15 ± 0.02 μg protein/egg and 0.24 ± 0.03 μg cholesterol/egg, for cRNA-injected isoosmotic oocytes 0.17 ± 0.03 μg protein/egg and 0.27 ± 0.04 μg cholesterol/egg, and for hypoosmotic oocytes 0.18 ± 0.02 μg protein/egg and 0.26 ± 0.04 μg cholesterol/egg. For cyclodextrin (10 mM for 60 min), the light membranes contained 0.08 ± 0.03 μg protein/egg and 0.11 ± 0.02 μg cholesterol/egg ($n = 5$ for each group).

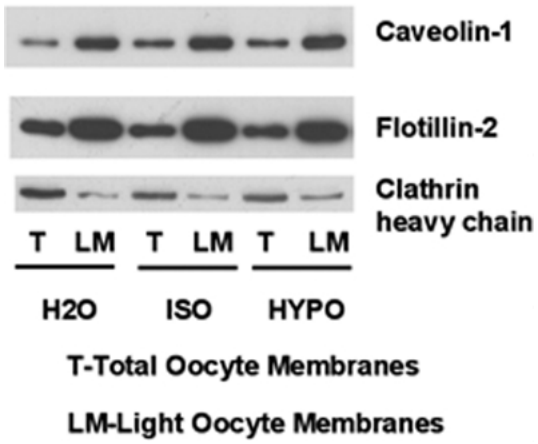

Fig. 5. Presence of lipid raft markers caveolin-1 and flotillin-2 and the heavy chain of clathrin in light membranes. Total and light membranes were isolated from (1) control, water-injected *Xenopus* oocytes, (2) *Xenopus* oocytes expressing skAE1 and incubated in isoosmotic ND109 (235 mOsm), or (3) hypoosmotic ND96 (210 mOsm). Total membranes (30 μg) and light membranes (10 μg) were loaded onto SDS polyacrylamide gel for electrophoresis and Western blot analysis for the presence of caveolin-1, flotillin-2, and the heavy chain of clathrin. Images are representative of three separate experiments.

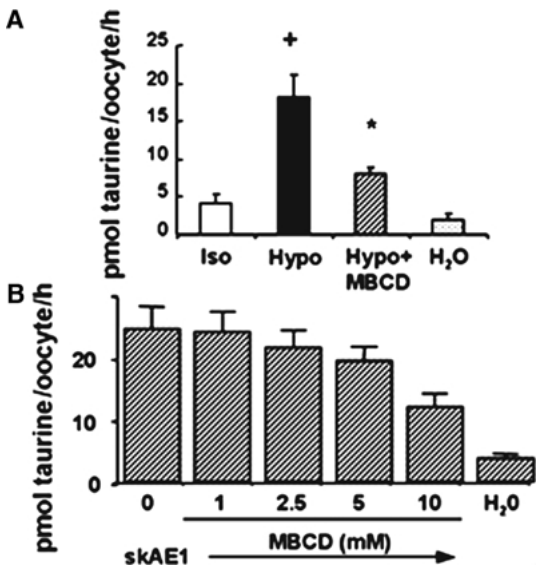

Fig. 6. Effects of MBCD on volume-stimulated taurine transport. (*A*) skAE1 was expressed in *Xenopus* oocytes and incubated in isoosmotic modified Ringer's (Iso, 235 mOsm), hypoosmotic ND96 (Hypo, 210 mOsm), or hypoosmotic ND96 in the presence of MBCD (10 mM for 60 min, MBCD). Control, water-injected oocytes were incubated in hypoosmotic ND96. Values are means ± standard error, $^{+}P < 0.01$ hypoosmotic vs. isoosmotic, $^{*}P < 0.01$ hypoosmotic vs. hypoosmotic plus cyclodextrin by analysis of variance. (*B*) Concentration dependence of MBCD inhibition of hypoosmosis-induced taurine uptake. Oocytes were injected with water or skAE1 cRNA and kept in ND109 for 3 days. MBCD was added 60 min prior to taurine uptake, and all uptakes were measured in hypoosmotic ND96. Values are expressed as means of all oocytes ± standard error.

The effects of cyclodextrin on tyrosine phosphorylation of skAE1 in total and light membranes and surface expression in these two membrane isolates were also investigated. Hypoosmotic exposure induced tyrosine phosphorylation of skAE1 when analyzed in either total or light membranes (third panels of left and right, Fig. 7), an effect which was also observed for surface-expressed skAE1 (biotinylated; fourth panels on left and right, Fig. 7). Surface-expressed skAE1 increased after hypoosmotic exposure in both total and light membranes (biotinylated; top and second panels on left and right, Fig. 7). The ability of hypoosmotic exposure to increase skAE1 in light membranes as well as to increase surface-expressed skAE1 (in both total and light membranes) was partially blocked by MBCD pretreatment. The skAE1 which did increase on the surface and in the light membranes demonstrated increased tyrosine phosphorylation, but phosphorylation was lower in oocytes treated with MBCD. The incomplete inhibition observed with MBCD correlated with a partial inhibition of hypoosmotic stimulation of taurine uptake. We intentionally did not treat oocytes for longer periods with cyclodextrin since severe cholesterol depletion might have induced some nonspecific effects on the oocytes.

Discussion

Skate erythrocytes, unlike their mammalian counterparts, express three anion exchanger isoforms [6]. When expressed in *Xenopus* oocytes, skAE1 and skAE2 mediate anion exchange and may confer taurine permeability [14, 15]. We focused on skAE1 since in the intact red cell, previous studies using a specific antiserum have demonstrated biochemical alterations and membrane localization of this isoform upon volume expansion [24–28]. The oocyte heterologous expression system allowed us to determine whether skAE1 alone, without interaction with other skAE isoforms, might demonstrate hypoosmosis-induced skAE1 alterations which correlate with increased taurine permeability in skate red blood cells. We do not know whether in the intact red cell skAE1 and other isoforms interact. Under isoosmotic conditions, skAE1 is found to a significant degree as dimers. Since we do not have a good antibody to probe skAE2 presently, we do not know if there is association of different AE isoforms. Therefore, the oocyte expression system, which demonstrated that skAE1 may mediate this function alone and which demonstrated similar regulation, is quite important. Future studies in both the skate erythrocyte as well as the oocyte expression system will help to elucidate the different abilities and regulation of the varying AE isoforms.

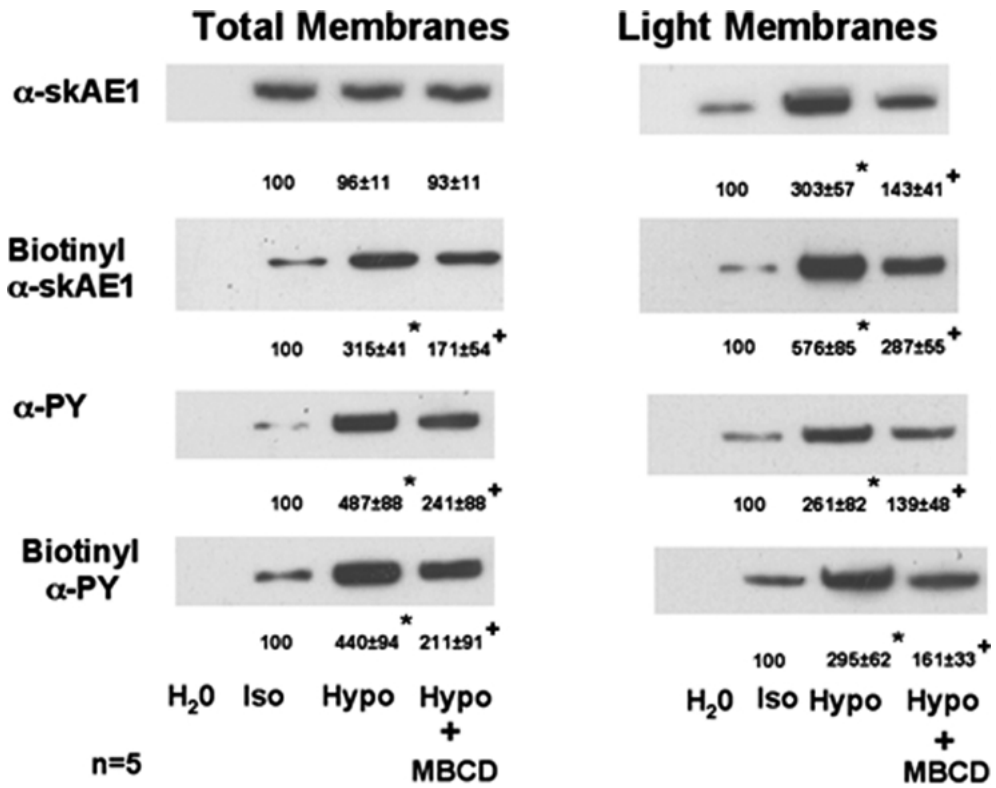

Fig. 7. Effect of MBCD-mediated cholesterol depletion of skAE1 localization and tyrosine phosphorylation following hypoosmotic exposure. Water- or skAE1 cRNA-injected oocytes were kept in ND109 for 3 days and then, when appropriate, treated with 10 mM MBCD for 60 min prior to transfer to hypoosmotic ND96 for 60 min. Oocytes were then biotinylated as described in Materials and Methods at 4°C for 30 min. skAE1 as well as its state of tyrosine phosphorylation (PY) were analyzed in both total membranes (*all panels on left*) and light membranes isolated on a discontinuous sucrose gradient (*all panels on right*). skAE1 was immunoprecipitated and analyzed for skAE1(*top panels on right and left*) or phosphotyrosine (*third panels down on right and left*) using anti-skAE1 or antiphosphotyrosine antibodies and Western blotting. A portion of the immunoprecipitation was further incubated with streptavidin agarose; therefore, only skAE1 at the surface was isolated. This surface-expressed skAE1 was also analyzed for the abundance of skAE1 and its level of tyrosine phosphorylation. Images are representative of four separate experiments. In all cases, isoosmosis was set to 100 and densitometry was performed, with increases based on fold increases over this value for each condition. $^{*}P < 0.05$ iso and $^{+}P < 0.01$ hypo vs. iso and hypo + MBCD vs. hypo alone, both comparisons by paired Student's *t*-test.

It is of interest that skAE1 activation appears to be regulated similarly in the oocyte and the red cell. A large number of simultaneous events occur upon volume expansion, and a number of biochemical pathways may be activated. Not all are likely needed for regulation of participation of skAE1 in increasing taurine permeability. The oocyte expression system has allowed us to better establish and validate the role of tyrosine phosphorylation and redistribution and localization in membrane microdomains.

Tyrosine kinases have been shown to increase the activity of other channels as part of the cell's volume regulatory mechanism [2, 35, 36, 38]. Tyrosine phosphorylation may increase the activity of osmosis-sensitive channels by inducing exocytosis of the transporter from an intracellular pool of vesicles [2] or by directly phosphorylating the channel and altering its structure and thereby potentiating its activity [38]. Additionally, tyrosine phosphorylation has been noted as an important regulatory event in the RVD of epithelial cells [33] and could be involved in the regulation of a number of channels and transporters activated upon volume expansion.

In addition to phosphorylation regulation of channel function, localization in different membrane microdomains appears to play a role both in oocytes as well as in the intact erythrocyte. Not only might channels or carriers be directed to membrane domains upon volume expansion but regulatory and/or signaling proteins could move in and out of light membrane/lipid raft regions. This indeed appears to be one mechanism of regulating the activity of transport proteins involved in volume regulation in a number of cell types [2, 8, 10, 17, 18, 31, 32, 34]. In the present study, the inhibitory effects of MBCD on the volume-sensitive organic osmolyte channel are consistent with the idea that lipid rafts are involved in regulating the activity of the channel.

Interestingly, treatment with pervanadate, a protein phosphatase inhibitor, potentiated the

activity of the volume-sensitive organic osmolyte channel in the absence of a stimulus (hypoosmotic stress). In unstimulated, isoosmotic conditions, oocytes may exhibit higher phosphatase activity, keeping more skAE1 in the unphosphorylated rather than the phosphorylated state, decreasing the open probability of the channel. Inhibition of the protein phosphatase may increase the phosphorylation of skAE1 toward levels comparable to those observed with hypoosmotic stimulation. Thus, the open probability of the channel is increased even in the absence of hypoosmotic stress. The lack of potentiation with pervanadate under hypoosmotic conditions may be due to the lack of substrate, unphosphorylated skAE1.

In summary, *Xenopus* oocytes expressing only one isoform of the skate erythrocyte anion exchanger, skAE1, demonstrate hypoosmosis-induced taurine permeability which bears much similarity to that of intact red cells, where mRNA for all three isoforms is expressed. The present results cannot rule out a potential role of skAE2 and/or skAE3 in hypotonically stimulated taurine transport previously observed in skate erythrocytes. However, they do indicate that skAE2 and skAE3 are not absolutely required for channel activation. The skAE1 protein appears to be sufficient to support hypoosmotically stimulated taurine transport through the volume-sensitive organic osmolyte channel. Also, the hypoosmotic stress-induced mobilization of skAE1, into light membranes and the cell surface, as well as tyrosine phosphorylation of skAE1 that occur in the oocyte support the biochemical events that are believed to be involved in the activation of the channel in the intact red cell.

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