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# Rho, Rho-kinase, and the actin cytoskeleton regulate the $Na^+$–$H^+$ exchanger in sea urchin eggs

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**Abstract**

At fertilization, the sea urchin egg undergoes an internal pH ($pH_i$) increase mediated by a $Na^+$–$H^+$ exchanger. We used antibodies against the mammalian antiporters NHE1 and NHE3 to characterize this exchanger. In unfertilized eggs, only anti-NHE3 cross-reacted specifically with a protein of 81-kDa, which localized to the plasma membrane and cortical granules. Cytochalasin D, C3 exotoxin (blocker of RhoGTPase function), and Y-27632 (inhibitor of Rho-kinase) prevented the $pH_i$ change in fertilized eggs. These inhibitors blocked the first cleavage division of the embryo, but not the cortical granule exocytosis. Thus, the sea urchin egg has an epithelial NHE3-like $Na^+$–$H^+$ exchanger which can be responsible for the $pH_i$ change at fertilization. Determinants of this $pH_i$ change can be: (i) the increase of exchangers in the plasma membrane (via cortical granule exocytosis) and (ii) Rho, Rho-kinase, and optimal organization of the actin cytoskeleton as regulators, among others, of the intrinsic activity of the exchanger.


*Keywords:* Egg; Fertilization; $pH_i$; $Na^+$–$H^+$exchange; Rho; Rho-kinase; Cytoskeleton; Exocytosis; Sea urchin

Sea urchin egg activation by sperm involves biophysical and biochemical events that are fundamental for development [1]. These events include a $Ca^{2+}$-dependent cortical granule exocytosis [2], reorganization of cortical actin cytoskeleton [3,4], and a $Na^+$-dependent increase in the cytoplasmic pH ($pH_i$) [5,6], among others. $pH_i$ regulates protein synthesis during early development [7,8]. It has been suggested that $pH_i$ elevation is sustained by a plasma membrane $Na^+$–$H^+$ exchanger [9,10]. Phorbol esters increase the $pH_i$ of unfertilized sea urchin eggs, suggesting that protein kinase C (PKC) may regulate the presumed $Na^+$–$H^+$ exchanger [10]; its regulation is, however, not fully understood. $Na^+$–$H^+$ exchangers (NHE) are membrane proteins that catalyze the exchange of intracellular $H^+$ for extracellular $Na^+$ ions [11]. In mammals, these transporters participate in $pH_i$ homeostasis, and in transient alkalinization evoked by agonists. Nine isoforms have been described in mammals and designated NHE1 through NHE9 [11]. They share a highly hydrophobic N-terminal domain. The C-terminal of the exchangers is hydrophilic and is located in the cytoplasm; it constitutes the domain of action for NHE regulators [12]. The NHE isoforms distribute among different subcellular compartments or are tissue-specific [12–16] and exhibit specific control mechanisms. For instance, NHE1 and NHE3 can be regulated by RhoGTPases as well as Rho-kinase—a Rho effector—and the cytoskeleton [17–20]. RhoA is present in the sea urchin egg [21,22]. The C3 exotoxin from *Clostridium botulinum*, which inactivates RhoGTPases [23], and cytochalasin D, which perturbs the actin cytoskeleton, both inhibit protein synthesis in sea urchin eggs [24]. In both cases, inhibition of protein synthesis is overcome by an artificial

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alkalinization. From these results, it has been hypothetized that optimal actin organization and Rho are required for the egg $Na^+$–$H^+$ exchange activity.

The aim of this study was to identify the $Na^+$–$H^+$ exchanger of the sea urchin egg that is responsible for the $pH_i$ change at fertilization. In particular, we examined proteins related to the mammalian NHE1 and NHE3 isoforms because they are active transporters of the plasma membrane in most cellular types (NHE1) and epithelial cells (NHE3). For this study, Western blotting and immunocytochemistry were performed using antibodies against the C-terminus of NHE1 and NHE3. We also analyzed whether at fertilization the exchange activity was controlled by the cytoskeleton, RhoGTPase, and Rho-kinase (ROCK) using the ''specific'' inhibitors cytochalasin D, C3 exotoxin, and Y-27632, respectively.

## Materials and methods

*Gamete collection*. *Strongylocentrotus purpuratus* sea urchins were from the bay of Ensenada (Baja California, Mexico). Eggs and dry sperm were obtained as indicated in [22].

*Chemicals*. [$^{32}$P]NAD and [$^{32}$P]ATP (1000 Ci/mmol) were obtained from Amersham. 2,7-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethylester (BCECF-AM) was obtained from Molecular Probes. Stock solutions of cytochalasin D and latrunculin B (Sigma) were prepared in DMSO. Y-27632 (Calbiochem) was dissolved in double distilled water. C3 exotoxin from *Clostridium botulinum* (Upstate Biotechnology) was prepared in PBS/glicerol (50%). Materials for electrophoresis were from Bio-Rad. Monoclonal antibodies against the C- terminus of porcine NHE1 and rabbit NHE3 were from Chemicon International. Anti-mouse IgG conjugated with Cy3 (for immunofluorescence) or horseradish peroxidase (for Western blotting) were from Jackson Immunoresearch. All other chemicals were from Sigma.

*Egg activation and first cleavage division*. Activation of eggs by sperm was evaluated by the appearance of the fertilization envelope [24], 10 min after insemination. To score the percentage of embryos that reached the first cell division, fertilized eggs, pretreated or not with different agents, were left in the dark (15 °C and for 1.5–2 h) and observed under the microscope.

*Egg fractionation*. Eggs were washed by centrifugation (2000*g*, 10 min) with artificial seawater (ASW), followed by resuspension in lysis buffer (50 mM Tris–HCl, 20 mM Hepes, EDTA 1 mM, 5 μg/ml antipain, 5 μg/ml leupeptin, 5 μg/ml pepstatin A, 5 μg/ml soybean trypsin inhibitor, and 10 mM NaF, pH 7.5) and homogenization. The extract was cleared by centrifugation at 2000*g* for 10 min; the supernatant was recovered and ultracentrifuged (100,000*g*, 1 h) to obtain whole membranes and the soluble fraction. All samples were saved and stored at −70 °C until use. Egg cortices, plasma membranes, and cortical granules were prepared as described [22].

*Western blotting and immunofluorescence*. For immunoblotting, the protocol was the same as indicated in [24], using 50–75 μg cellular protein and an antibody dilution of 1:1000. Cross-reactivity was revealed by chemiluminiscence. Indirect immunofluorescence of eggs and embryos was recorded as follows: eggs (5% v/v) were pretreated for 30 min with 0.5 mM EDTA before insemination. The cell suspension was divided into two aliquots. One of these was fixed for 90 min with 2% paraformaldehyde in ASW (pH 8) and other was first mixed with sperm (1:16 dilution of dry sperm) and incubated at 15 °C for 10 min before fixation. Both samples were processed for immunostaining as described in [24], with anti-NHE1 or anti-NHE3 at a 1:250 dilution. Anti-mouse IgG (1:100) conjugated with Cy3 was used. Images were acquired with confocal Zeiss LSM510 Meta and a Zeiss Axiovert 200 M microscope, and analyzed using the Image Examiner software.

*$pH_i$ measurements*. $pH_i$ was measured in eggs in suspension using the fluorescence indicator BCECF [25]. Briefly, eggs (4000 cells) were suspended in 125 μl ASW (pH 7.0) containing the permeable form of the probe (15 μM BCECF-AM) and incubated in the dark for 1 h at 17 °C. External BCECF-AM was removed by centrifuging the eggs (500*g*, 2 min at 4 °C) and washing twice. Ten microliters of BCECF-loaded eggs was added to 0.8 ml of regular ASW. After mixing for 2 min, fluorescence was recorded (490/440 nm for excitation and 520 nm for emission) in a SLM Aminco 8000 spectrofluorimeter keeping the temperature at 17 °C. Under these conditions autofluorescence was less than 2%. Sperm (40 μl of a 1:16 dilution in CaSW, pH 7.0) were added where indicated. As reported [25], these conditions allowed the determination of the normal $pH_i$ change that eggs undergo at fertilization. The fluorescence versus time was normalized considering the fluorescence change evoked by sperm in the absence of inhibitors to be 1.0.

*Other assays*. Acrosome reaction (AR) of sperm was scored as described in [26]. Rho was identified by [$^{32}$P]ADP-ribosylation with C3 exotoxin from *C. botulinum* [22]. $Na^+/K^+$-ATPase assay was determined as the difference in Pi released in the presence and absence of 1 mM ouabain [27].

## Results

### *Characterization and subcellular localization of the sea urchin egg $Na^+$–$H^+$ exchanger*

Western blotting of whole membranes from unfertilized eggs was performed using two antibodies against the carboxy-terminus of NHE1 and NHE3. The NHE3 antibody cross-reacted with an egg protein of 81 kDa (Fig. 1A); this molecular mass is in the range (80–87 kDa) for mammalian NHE3 [28]. No immunoreactivity was observed when anti-NHE3 was omitted in the assay (Control 1) or when it was pre-adsorbed with epithelial membranes from rabbit

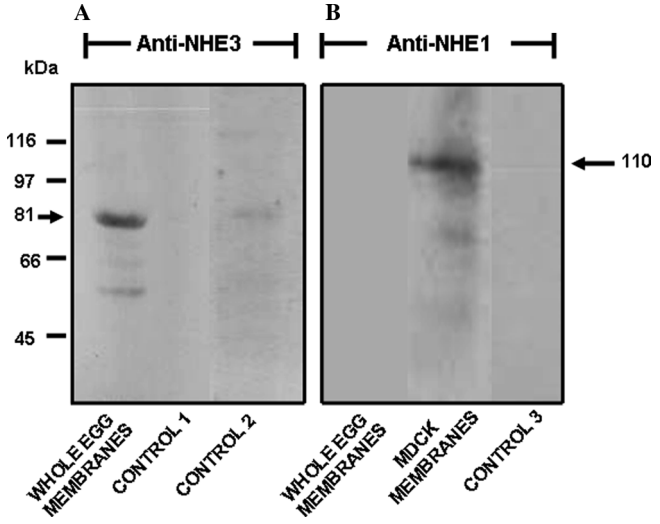


Fig. 1. Western blot of the sea urchin egg $Na^+$–$H^+$ exchanger. Monoclonal antibodies against rabbit NHE3 and porcine NHE1 were used for immunoblotting of whole egg membranes. Control 1, anti-NHE3 antibody was omitted. Control 2, anti-NHE3 was pre-adsorbed with epithelial membranes from rabbit intestine. Whole MDCK membranes were tested as positive control of anti-NHE1 immunoreactivity; its respective control is without the primary antibody (Control 3). Blots were stained with amide black to verify protein (75 μg/lane) loading. Results shown are representative of three independent experiments.