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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

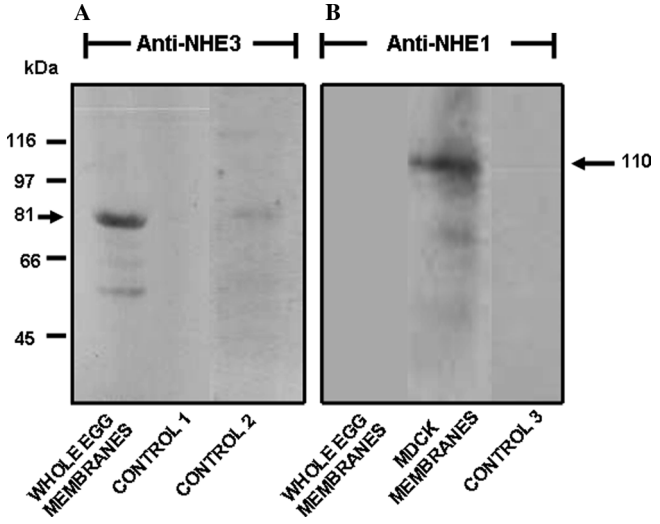


Fig. 1. Western blot of the sea urchin egg $Na^+$–$H^+$ exchanger. Monoclonal antibodies against rabbit NHE3 and porcine NHE1 were used for immunoblotting of whole egg membranes. Control 1, anti-NHE3 antibody was omitted. Control 2, anti-NHE3 was pre-adsorbed with epithelial membranes from rabbit intestine. 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.

intestine (Control 2). Microsomes and soluble proteins did not react with anti-NHE3 (not shown). These results indicate that the observed cross-reactivity is authentic and, therefore, the sea urchin egg has a NHE3-like antiporter. Anti-NHE1 antibody showed no immunoreactivity in whole egg membranes (Fig. 1B), despite testing several dilutions below 1:1000. A positive control showed that anti-NHE1 immunodetected a MDCK membrane protein of the expected molecular weight (~110 kDa) for NHE1 [29], suggesting the absence of a NHE1-related isoform in the sea urchin egg.

The sea urchin egg cortex includes the extracellular viteline layer, the plasma membrane, and the subplasmalemmal region which contains cortical granules and actin [2,3]. Fig. 2A shows the presence of the NHE3-like antiporter in isolated cortices. This protein localized to the cortical granules and plasma membranes prepared from these cortices (Fig. 2A). As equivalent amounts of protein were used, densitometric analysis of signal intensities shown in Fig. 2A demonstrated that cortical granules contain higher levels of the NHE3-like protein than plasma membranes (Fig. 2C). Rho was used as a marker of cortical granules in order to verify the relative purity of these vesicles [22]. The autoradiography shows the expected [$^{32}$P]ADP-ribosylation of Rho by C3 exotoxin in egg cortices as well as in isolated cortical granules (Fig. 2B). As observed before, plasma membranes did not exhibit significant radiolabeling. In addition, we measured $Na^+/K^+$-ATPase activity as a plasma membrane marker to estimate the contamination of cortical granules with plasma membranes. Of the total ATPase inhibitable by 1 mM ouabain, less than 5% was in cortical granules, while the rest was detected in plasma membranes (not shown), precluding possible contamination of cortical granules by plasma membranes. To further confirm the cellular distribution of the egg exchanger, unfertilized eggs were processed for immunofluorescence. The NHE3 antibody revealed immunostaining in the peripheral region of the egg (Fig. 3A), which is consistent with results of Fig. 2. Additionally, the distribution of the exchanger in embryos at 10 min-postfertilization, when cortical granule exocytosis is complete, did not show detectable changes with respect to its localization in unfertilized eggs, indicating that this transporter remains in the cellular periphery and is not internalized. Mammalian NHE3 undergoes phosphorylation by protein kinase A [30]. Fig. 3B shows that the exchanger did not modify significantly its electrophoretic mobility, suggesting that phosphorylation does not occur, or it does at an undetectable extent by this technique.

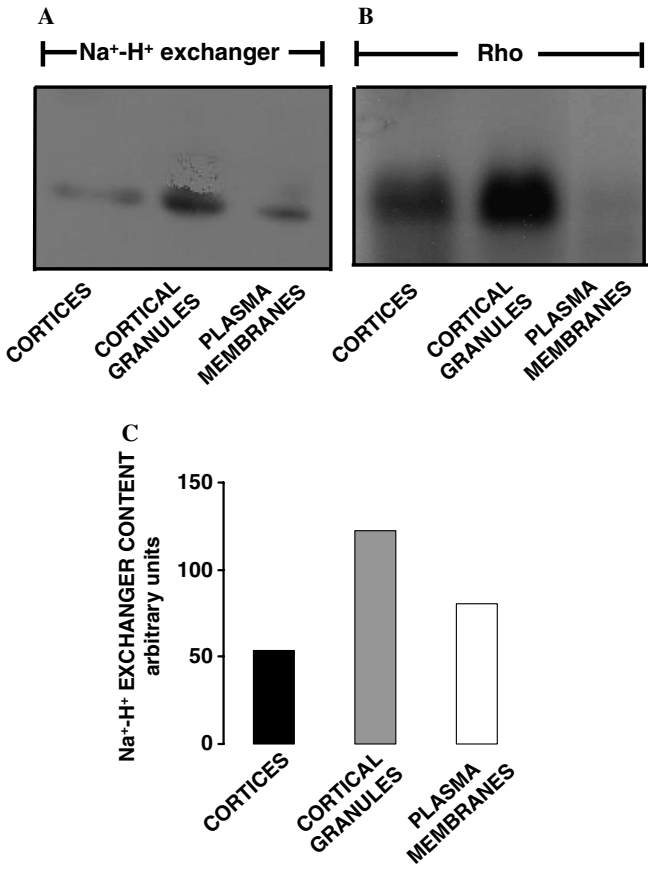


Fig. 2. The egg $Na^+$–$H^+$ exchanger locates to the cortical granules and plasma membrane. The presence of the egg exchanger and RhoGTPase (cortical granule marker) was assayed in cortices, cortical granules, and plasma membranes obtained from unfertilized eggs. (A) Immunoblot for anti-NHE3. (B) [$^{32}$P]ADP-ribsosylation of Rho with C3 exotoxin (autoradiography). (C) Densitometric analysis of results shown in (A). Equivalent amounts of cellular protein (75 μg protein) were used under each condition. Results are representative of three separate experiments.

### *Effect of cytochalasin D, C3 exotoxin, and Y-27632 on sperm-induced $pH_i$*

Mammalian NHE3 is sensitive to actin cytoskeleton destabilizers [20]. $Na^+$–$H^+$ exchange activity was measured using the $pH_i$-sensitive fluorescence of BCECF in eggs washed after incubation with 60 μM cytochalasin D for 15 min. As reported earlier [6,25], nontreated eggs respond to sperm by increasing their $pH_i$ after a lag period of 1–1.5 min (Fig. 4A). Eggs pretreated with cytochalasin D did not increase their $pH_i$ upon fertilization. To discard external effects of cytochalasin D on sperm, they were recovered and their AR evaluated at the end of the experiment. Under these conditions, over 80% sperm underwent acrosomal exocytosis (not shown). We also examined the effects of latrunculin B, which is chemically unrelated to cytochalasin D and acts by forming complexes with G-actin, thus reducing the pool available for polymerization [31]. Latrunculin B inhibited 80% of the $pH_i$ increase induced by sperm (not illustrated).

The state of actin polymerization is controlled by members of the Rho family [32]. These proteins lose their function as they are ADP-ribosylated by C3 exotoxin [23]. Additionally, Rho regulates some forms of the mammalian $Na^+$–$H^+$ exchanger [17–19]. In sea urchin eggs, C3 perturbs the actin cytoskeleton [24]. Thus, C3 was used to examine the relationship between the Rho function and

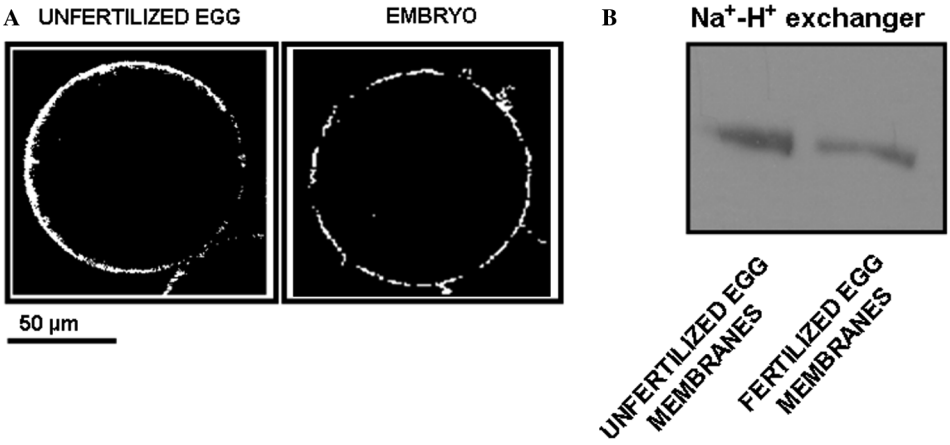


Fig. 3. Immunolocalization and Western blot of the $Na^+$–$H^+$ exchanger in eggs and embryos. (A) Unfertilized eggs and embryos (10 min postfertilization) were processed for immunostaining with anti-NHE3 antibody and samples analyzed by confocal microscopy. (B) Anti-NHE3 antibody was used for immunoblotting of crude membranes from unfertilized eggs and embryos. The amounts of cellular protein loaded in the gel were the same. Results shown are representative of three experiments.

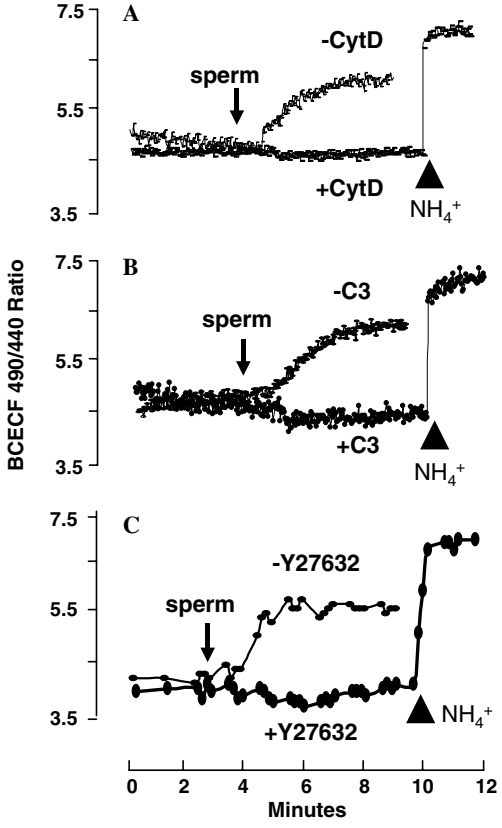


Fig. 4. Effect of cytochalasin D, C3 exotoxin, and Y-27632 on the sperm-induced $pH_i$ increase in eggs. BCECF-loaded eggs were preincubated with 60 µM cytochalasin D, 0.5 ng/µl C3 or 60 µM Y-27632, washed with artificial seawater and inseminated. Where indicated (arrowheads), 20 mM $NH_4Cl$ was added to eggs treated with the respective inhibitor to ensure that the dye still responds to $pH_i$ changes. Representative traces, of at least three separate experiments, are shown.

the $Na^+$–$H^+$ exchange activity. BCECF-loaded eggs were preincubated without (control) and with 0.5 ng/µl C3. Control (no toxin) eggs normally alkalinized at fertilization, while treatment with C3 drastically inhibited the $pH_i$ increase evoked by sperm (Fig. 4B). Separate experiments revealed that C3 does not inhibit AR. Moreover, sperm recovered after interacting with BCECF-loaded eggs appeared acrosome reacted. Among the known effectors of Rho is the serine/threonine kinase, ROCK [33]. The possible involvement of ROCK in the regulation of the sea urchin egg $Na^+$–$H^+$ exchange activity was tested using the compound Y-27632, a seemingly selective inhibitor of this kinase [34]. The experiment was conducted under the same conditions used to test the C3 effect. Fig. 4C shows the marked inhibition caused by 60 µM Y-27632 on the $pH_i$ rise. Separate experiments showed that the egg jelly induced sperm AR is sensitive to Y-27632 and to the more potent ROCK antagonists, H-1152 (manuscript in preparation). Although eggs pretreated with Y-27632 were washed before $pH_i$ recording and sperm addition, low amounts of extracellular Y-27632 could be inhibiting the egg $pH_i$ rise by blocking the sperm AR. This was ruled out by confirming that sperm taken from the cuvette had undergone the AR 5 min after insemination (not shown). As controls, 20 mM ammonia was added to eggs treated with inhibitors to ensure that the plasma membrane remained intact and that the fluorescent probe was still sensitive to $pH_i$ changes (arrowheads in Fig. 4). In summary, assuming a $\Delta pH_i$ value of 1.0 for nontreated eggs, results with three different egg lots performed as in Fig. 4A–C indicated that $\Delta pH_i$ ($\pm$SD) in presence of cytochalasin D, C3, and Y-27632 was $0.14 \pm 0.03$, $0.17 \pm 0.06$, and $0.20 \pm 0.02$, respectively. These compounds also reduced the first cleavage division to $9.66 \pm 3.05\%$, $12.33 \pm 6.50\%$, and $17 \pm 4.32\%$, respectively. Y-27632, as C3 [24] and cytochalasin D [35], allowed the formation of the fertilization envelope indicating that the inhibitor, at the concentrations used, acts downstream of the cortical granule exocytosis. Phallodin-staining of eggs treated with Y-27632 revealed a massive disruption of cortical actin filaments, as judged by the marked

decrease in fluorescence intensity with respect to the basal level of F-actin of control eggs (results not shown). The effect of Y-27632 resembles the modifications caused by C3 and cytochalasin D reported earlier.

## Discussion

Our findings indicate that the sea urchin egg has a $Na^+$–$H^+$ antiport (81 kDa) immunochemically related to mammalian NHE3. This is presumably the transporter supporting the cytoplasmic alkalinization during egg activation and behaves as the mammalian NHE3 in several ways. First, optimal $Na^+$–$H^+$ exchange activity requires basal levels of F-actin in the egg cortex, as observed in mammalian NHE3 activity [35]. This result is consistent with the observation that acid release by activated eggs is blocked by cytochalasin B [36]. Interestingly, a cytoskeleton–exchanger interaction is suggested by co-sedimentation of the 81 kDa protein with the actin cytoskeleton in a detergent (Triton X-100)-insoluble fraction of unfertilized eggs (results not shown). Second, the egg exchanger also shares regulatory characteristics with epithelial NHE3 with respect to the influence of RhoGTPase on the ion exchange activity [17–19]. It is likely that RhoGTPase regulation of the egg $Na^+$–$H^+$ exchanger is linked to the functional modulation the actin cytoskeleton that exerts on this transporter, considering that this GTPase influences the cytoskeletal organization [32]. Last, the egg exchanger is related with mammalian NHE3 in that its activation at fertilization is mediated by ROCK, one of the Rho effectors in many cellular types [33]. This conclusion is based on the fact that the $pH_i$ increase elicited by sperm is blocked by the ROCK inhibitor Y-27632, which also inhibits NHE3 activity [19]. Apparently, there is no direct phosphorylation of the egg exchanger [19]. Therefore, the intrinsic activity of the egg $Na^+$–$H^+$ exchanger is, in part, regulated by the cytoskeleton and the Rho/Rho-kinase proteins but it may be influenced by other factors as PKC [10] and lipid rafts [37]. The distribution of the exchanger between the plasma membrane and cortical granules in unfertilized eggs suggests an additional mode of regulation. The proposed model is that the exchangers residing in the plasma membrane may have a role in controlling the resting $pH_i$, protecting the unfertilized egg against intracellular acidosis produced by basal metabolism. Upon fertilization, cortical granule exocytosis would result in insertion of exchangers in the plasma membrane, increasing the number of copies in this compartment and, consequently, accounting for the intracellular alkalinization. Interestingly, mammalian NHE3 cycles between the plasma membrane and endosomes to control the ion exchange activity [38]. Although an egg undergoes a $pH_i$ increase upon insemination, the physiological significance of this event is still controversial. In one hand, some reports indicate that this $pH_i$ increase is crucial in accelerating the rate of protein synthesis [8,9]. On the other, $pH_i$ seems to be not important [39]. Therefore, the mechanisms underlying protein synthesis activation may depend on the $pH_i$ but other factors should be considered.

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