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Phorbol Ester Activation of an NHE-Like Electroneutral Na⁺/H⁺ Antiporter in Isolated E-Cells of Lobster (Homarus americanus) Hepatopancreas

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ABSTRACT The basolateral membrane of Atlantic lobster (Homarus americanus) epithelium possesses an electroneutral Na⁺/H⁺ antiporter that functionally resembles members of the vertebrate NHE family. Regulatory mechanisms of this antiporter in purified hepatopancreatic E-cell suspensions, produced with a centrifugal elutriation technique, were investigated. Suspensions routinely consisted of greater than 95% E-cells displaying greater than 90% viability. Intracellular pH (pHi) was monitored by loading cells with the fluorescent dye 2’,7’-bis(carboxyethyl)-5,6-carboxyfluorescein (BCECF), and placing suspensions in a spectrofluorometer. Recovery from induced acid-loading was mediated by a Na⁺-dependent, dimethylamiloride-sensitive proton efflux. Antiport activation was a sigmoidal function of pHi at values below 7.0. Addition of 20 nM phorbol 12-myristate 13-acetate (PMA) to cells suspended in a lobster physiological saline (pH o = 7.4) increased pHi from 7.2 to 7.5 over a 10-min interval. Phorbol ester-induced activation of the Na⁺/H⁺ antiporter was due to an increased affinity for internal H⁺ (apparent pK was shifted toward more alkaline values) at the level of an internal H⁺-binding allosteric modifier site. No effect was observed when cells were exposed to 2 µM 8-Br-cAMP. Phorbol ester activation of the lobster NHE-like Na⁺/H⁺ antiporter was inhibited by 10 nM bisindoylmaleimide I, a potent protein kinase C inhibitor. These results taken together suggest a remarkable conservation of Na⁺/H⁺ antiporter across phyla. J. Exp. Zool. 281:97–108, 1998. © 1998 Wiley-Liss, Inc.

Na⁺/H⁺ antiport has been documented in nearly every eukaryotic cell investigated. Na⁺/H⁺ antiporters are transmembrane-spanning transport proteins that catalyze the net electroneutral exchange of one intracellular H⁺ for one extracellular Na⁺ (Grinstein, '88) and are inhibited by amiloride and its 5-amino substituted derivatives (Aronson, '85). Several isoforms of the Na⁺/H⁺ antiporter have been cloned from vertebrate species, the first being referred to as NHE1 (Sardet et al., '89), followed by NHE2 (Tse et al., '91), NHE3, and NHE4 (Orlowski et al., '92). In nonepithelial cells, NHE1 is solely present and functions as a “housekeeping” protein participating in intracellular pH and volume regulation and transepithelial Na⁺ transport.

Comparative investigation into Na⁺/H⁺ antiport in invertebrate species has resulted in the identification of two separate types of exchange mechanisms. Initially studies utilizing brush-border membrane vesicles (BBMV) and either radio-isotopic tracers or acridine orange methodologies described an electrogenic 2Na⁺/1H⁺ antiporter in lobster and prawn hepatopancreas (Ahearn and Clay, '89; Ahearn et al., '94), crab gill (Shetlar and Towle, '89), and crustacean antennal gland cell plasma membranes (Ahearn and Franco, '90). This electrogenic antiporter was amiloride-sensitive and could accommodate Ca²⁺ as a substrate in lieu of two Na⁺ ions. Recent investigations into Na⁺/H⁺ antiport on the basolateral membrane of Atlantic lobster hepatopancreas epithelial cells disclosed an electroneutral Na⁺/H⁺ antiporter that functionally resembled members of the vertebrate NHE family (Duerr and Ahearn, '96).

Intracellular pH regulation is extremely important because a variety of cellular processes are pH-sensitive, such as the modulation of transepithelial solute transport, enzyme function, cell growth, and differentiation. NHE1 can detect changes in cytoplasmic pH by virtue of an intracellular-facing, allosteric H⁺-binding modifier...
site(s) (distinct from Na\(^+\) and H\(^+\) transport sites) that activates Na\(^+\)/H\(^+\) exchange when occupied by intracellular H\(^+\) (Aronson et al., '82). Activation of Na\(^+\)/H\(^+\) exchange may also result from phosphorylation of the antiporter or an ancillary protein by soluble intracellular kinases such as protein kinase C. Protein kinase C (PKC) is activated by diacylglycerol, a product of the hydrolysis of inositol phospholipids, or by tumor-promoting phorbol esters. Grinstein et al. ('85) characterized a phorbol-ester-induced increase in Na\(^+\)/H\(^+\) exchange brought about by a change in the cytoplasmic pH sensitivity of the antiporter. Growth factors such as EGF (epidermal growth factor) also stimulate Na\(^+\)/H\(^+\) exchange (Moolenaar et al., '83). The affinity of the allosteric modifier site for H\(^+\) is enhanced in the presence of alpha-thrombin or insulin (L'Allemain et al., '84; Paris and Pouyssegur, '84), suggesting a role for Na\(^+\)/H\(^+\) antiport in intracellular alkalinization prior to DNA synthesis and cell growth.

Very little is known regarding cellular mechanisms of hormonally mediated regulation of plasma-membrane-bound proteins in crustacea. Second-messenger mechanisms similar to those described for vertebrates have been reported in studies of crustacean hyperglycemic hormone (CHH). CHH elevates hemolymph glucose levels, and this action may be mimicked by cyclic nucleotides, which suggests the involvement of protein kinases and subsequent phosphorylation of cell proteins (Sedlmeier, '85).

This report details a comparative approach to delineating mechanisms of regulation of the Atlantic lobster (Homarus americanus) electroneutral Na\(^+\)/H\(^+\) antiporter present in basolateral membranes of hepatopancreas epithelial cells. The electroneutral Na\(^+\)/H\(^+\) exchanger present in lobster (lobster NHE) likely performs housekeeping functions and is probably expressed in all hepatopancreas cell types. The lobster hepatopancreas is an intestinal diverticulum and consists of four cell types: E, F, R, and B (Brunet et al., '94), each of which plays a specific physiological role. E-cells, located in the distal tips of the blind-ended sacs that comprise the hepatopancreas ultrastructure, are embryonic stem cells that differentiate into the remaining three cell types.

This study utilized purified E-cell suspensions prepared using a novel centrifugal elutriation technique and the intracellular pH-sensitive reporter dye 2',7'-bis-carboxy-ethyl-5,6-carboxyfluorescein (BCECF) to elucidate processes by which the electroneutral lobster NHE is regulated in vivo. The conclusions of this investigation include the identification of a Na\(^+\)/H\(^+\) antiporter on the plasma membranes of E-cells that is electroneutral, inhibited by dimethylamiloride and exogenous Li\(^+\), capable of mediating phorbol ester-induced intracellular alkalinization, and regulated by an internal H\(^+\)-sensitive activator site.

**MATERIALS AND METHODS**

**Animals**

Live intermolt Atlantic lobsters (Homarus americanus) were obtained from local commercial dealers in Hawaii and maintained in continuous-flow, refrigerated (10\(^\circ\)C), filtered-seawater tanks. Lobsters were maintained on diced squid for up to 10 days. Experiments were conducted only on lobsters that had not been fed for approximately 24 h to assure an evacuated hepatopancreas.

**Hepatopancreas cell dissociation**

Hepatopancreatic E-cell suspensions were prepared from fresh organs of individual (0.5 kg) lobsters. Hepatopancreatic tissue was quickly excised and placed into an isosmotic citrate buffer similar to that used in previous studies by Ahearn et al. ('83), consisting of (in mM): 27 Na-citrate, 443 NaCl, 5.6 NaH\(_2\)PO\(_4\), 1.5 KCl, 8.8 KH\(_2\)PO\(_4\), and 0.3 phenylmethylsulfonylfluoride (PMSF), pH 7.4. The tissue was manually minced for 5 min and then gently agitated for 30 min on an orbital shaker at room temperature. The resulting homogenate was filtered through a 200-µm nylon mesh to remove undisassociated tissue and large debris and centrifuged at 100g for 10 min with a Sorvall RC5C centrifuge. The supernatant was collected and used as a starting material.

**Centrifugal elutriation**

Centrifugal elutriation (see McEwen et al., '68) is a fast and gentle procedure for separating biological particles using the principle of a counterstreaming centrifuge. A concentrated suspension containing all four hepatopancreas cell types in LPS was aspirated into a 5-ml sterile syringe for injection into the elutriator. Using a buffer (LPS) flow rate of 5 ml/min and a rotor speed of 1,000 rpm (Beckman JA-6 rotor, Beckman J-21 centrifuge), a fraction was collected that comprised, on
average, an approximately 95% composition of E-cells. Dilute samples of E-cells were pelleted using a table-top centrifuge at maximum speed for 10 min and resuspended in LPS.

E-cells were identified visually by light microscopy at x400 magnification. E-cells or embryonic cells are uniformly approximately 50% the size of the other cell types found in the hepatopancreas and do not contain any specialized intracellular morphology or inclusions such as large vacuoles (B cells), lipid vesicles (R-cells), or extensive endoplasmic reticulum (F-cells) (Brunet et al., '94). Purity of preparation was also determined visually by counting the number of E-cells contained in a total population of elutriated cells (contaminating cells were always R cells) using an Improved Neubauer hemocytometer slide.

**Intracellular pH measurements using BCECF**

The fluorescent pH indicator BCECF-AM was loaded into cells by incubation for 20 min at room temperature in LPS containing 2.5 µM of the dye; cells were then washed by centrifugation (to remove excess and leaked dye) and diluted to a desired concentration. Cells were both counted and assayed for total protein concentration (BioRad). Fluorescence of labeled cell suspensions was measured in a Perkin-Elmer LS-5 spectrofluorometer connected to a recorder. Intracellular pH was measured as the ratio of emission at 520 nm after excitation at 490nm (pH and concentration sensitive) and 440 nm (concentration sensitive). The fluorescence ratio gives concentration-independent and pH-sensitive fluorescence measurements (Roos and Boron, '81). Two hundred µL of cell suspension were diluted into 1800 µL of test media (composition varied with experiment) for a total cell concentration of 21.3 ± 5.4 × 10⁸ cells/ml. Calibration of pH, versus fluorescence was determined using the high K⁺/nigericin technique (Thomas et al., '79).

Intracellular K⁺ concentration of E-cells was determined to be approximately 130 ± 4 mM. Briefly, a known quantity (cells/mL) of E-cells was diluted into deionized and distilled water to be assayed for K⁺ concentration using an atomic absorption spectrophotometer and K⁺ standards (n = 3). An average E-cell intracellular volume was calculated by measuring the major and minor axes of individual elliptically shaped E-cells (n = 30) using a BioRad confocal microscope and NIH-Image digital imaging software.

Cells were dye loaded in a modified LPS with 130 mM K⁺ containing 5 µM nigericin and injected into a cuvette containing LPS at varying pH and containing 0.5 µM nigericin. The ionophore maintains H⁺/H⁺ = K⁺/K⁺. This is accomplished when cells are suspended in an isosmotic saline containing 130 mM K⁺ (approximate cytoplasmic concentration) H⁺ and H⁺ equilibrate across the cell membrane (Fig. 1).

Determination of resting pH, in E-cells (pH = 7.2 ± 0.03) was accomplished by null-point analysis (Eisner et al., '89). Briefly, dye-loaded cells were exposed to a series of null pH solutions ranging from pH 6.8 to 7.6 consisting of varying concentrations of a weak base and a weak acid (ammonium/propionate). The general principle of the technique is that cells exposed to a combination of a weak acid and a weak base will experience a change in their pH, if the null solution is either above or below the actual resting pH. If the pH of the null solution is equal to the actual resting pH, then no change (null pH) will occur. In order to determine whether the changes of BCECF fluorescence were proportional to the change in pH, over the range required for experimental manipulations, the null technique was applied to pH values ranging from pH 6.4 to pH 7.6, using methods described in Boyarski et al. ('96a,b), and compared to the calibration curve obtained using the high K⁺/nigericin technique. In lobster hepatopancreatic E-cells, no difference was detected for pH values within the above experimental range and therefore no correction factor was necessary for the high K⁺/nigericin calibration curve.

Manipulation of pH, for the purposes of stimulating the Na⁺/H⁺ exchanger and examining the effect of pH on antiporter activation was accomplished using the method described by Grinstein et al. ('84). Briefly, cells were suspended in a choline⁺ medium containing (in mM): 470 choline chloride, 10.0 KCl, 2.0 CaCl₂, 6.7 MgCl₂, 30.0 HEPES, 10 D-glucose, pH 7.4. Acid loading could be accomplished by the addition of 0.5 µg/ml nigericin. When the desired pH, was reached, the effect of the ionophore was nullified by the addition of albumin (2.5 mg/ml final).

Determination of E-cell intracellular buffering power (β) was accomplished by NH₄⁺ titration as described in Strazzullo and Canessa ('90). NH₄Cl was added (2.5 mM final) to cells acidified to a desired level using the nigericin-albumin technique and suspended in choline⁺ medium. The pH was monitored and buffering power calculated as the change in [NH₄⁺] divided by the change in
Proton efflux was calculated at any given pH as H⁺ efflux = intracellular buffering power (mmol/L cell water · pH) divided by the product of the rate of pH recovery (pH/s).

Possible quenching of extracellular dye signal by drugs was assessed, and the fluorescence of extracellular BCECF was not affected by nigericin, phorbol 12-myristate, 13-acetate, bisindoylmaleimide I, 8-bromo-cAMP, or dimethylamiloride at the concentrations and instrument settings used in these experiments.

**Chemicals**

Bis-(carboxyethyl)carboxyfluorescein acetoxymethyl ester (BCECF-AM) was obtained from Molecular Probes, Inc. (Eugene, OR); dimethylamiloride (DMA) and nigericin were from Sigma Chemical Co. (St. Louis, MO); phorbol 12-myristate, 13-acetate, bisindoylmaleimide I, 8-bromo-cAMP, or dimethylamiloride at the concentrations and instrument settings used in these experiments.

**RESULTS**

**Calibration of fluorescence versus pHᵢ**

Resting intracellular pH was determined by null-point analysis as described in Materials and Methods. Purified E-cells were resuspended in a series of null solutions at variable pH. No alteration in the fluorescent signal, and hence pHᵢ, was recorded for null solutions approximating pH 7.2, whereas null solutions with pH < 7.2 resulted in E-cell alkalinization and null solutions with pH > 7.2 resulted in E-cell acidification. These results indicated that the resting pHᵢ of E-cells approximated 7.2. In order to address whether or not BCECF was accurately reporting pHᵢ in the range required for experimental purposes, null-point analyses were conducted at alkaline and acidic pHᵢ, and these data were compared with the fluorescence versus pHᵢ standard curve. Figure 1 represents the standard curve generated for fluorescence versus pHᵢ as determined by the high K⁺/nigericin technique described previously. Null-point analysis data derived at high and low pHᵢ agreed with those data obtained using the high K⁺/nigericin technique and therefore confirmed the reliability of BCECF and the standard curve in Figure 1 at all intracellular pH values E-cells experienced in the procedures described in this report.
**Determination of intracellular buffering capacity (β)**

Freshly purified lobster hepatopancreas E-cells were placed in a choline⁻ medium containing 0.5 μM nigericin and allowed to acidify to a desired pHᵢ. The pHᵢ was stabilized by the addition of albumin to the suspension. The addition of 2.5 mM NH₄Cl caused intracellular alkalinization, which was recorded immediately. The buffering power was then calculated as Δ[NH₄⁺]/ΔpHᵢ, assuming a pKᵢ of 9.21 and that NH₃ was distributed equally across the cell membrane. As shown by Grinstein et al. ('84), cell buffering power varied as a function of pHᵢ, with greater buffering power recorded for lower pHᵢ values. Intracellular buffering power of lobster E-cells varied linearly with pHᵢ (Table 1). Proton efflux could then be calculated using the following formula: 

\[ \text{H⁺ efflux} = \frac{\text{intracellular buffering power (mmol/L cell water · pH)}}{\text{product of the rate of pHᵢ recovery (pH/s)}} \]

**Effect of Li⁺ and monoclonal antibodies of 2Na⁺/1H⁺ exchange**

In order to distinguish between the two possible types of Na⁺/H⁺ exchange (electrogenic or electroneutral), the inhibitory efficacy of external Li⁺ and two monoclonal antibodies was assessed (Fig. 2). BCECF-loaded E-cells were suspended in standard LPS and acid-loaded with the addition of 30 mM propionate⁻ as described in methods. The presence of 10 mM Li⁺ significantly reduced (P < 0.05) the Na⁺-dependent return to baseline pHᵢ. Monoclonal antibodies (MAb 9 and Mab 11) generated against the brush-border 2Na⁺/1H⁺ antiporter functionally inhibit electroneutral exchange but are without effect on basolateral electroneutral exchange in membrane vesicle preparations (Duerr and Ahearn, '96). Neither antibody inhibited pHᵢ recovery in isolated E-cells, suggesting the sole presence of the electroneutral type of antiporter as described for lobster hepatopancreas basolateral membranes on E-cell plasma membranes.

**Kinetics of the E-cell Na⁺/H⁺ exchanger**

The dependence on extracellular Na⁺ by E-cell Na⁺/H⁺ exchange was quantitatively described by varying extracellular Na⁺ from 0 to 200 mM in a choline⁻ medium. E-cells were suspended in choline⁻ medium and acidified with 30 mM K⁺-propionate and pHᵢ monitored. Na⁺-dependent proton efflux was calculated as described in Materials and Methods over the first minute and could be described as a function of a distinct saturable carrier exhibiting Michaelis-Menten kinetics. The system could be quantitatively described by the following equation:

\[ J_H = (J_{\text{max}} \cdot [\text{Na}^+] / K_M + [\text{Na}^+]) \]

where \( J_H \) is the outward H⁺ flux in μmol/s · L cell water, \( J_{\text{max}} \) is the maximal carrier-mediated H⁺ flux in mmol/s · L cell water, \( K_M \) is the concentration of Na⁺ resulting in 1/2 \( J_{\text{max}} \) and [Na⁺] is the concentration of Na⁺ in mmol/L. The hyperbolic curve in Figure 3 represents the carrier-mediated Na⁺ uptake and suggests a 1:1 stoichiometry for Na⁺/H⁺ exchange.
exchange. Kinetic constants were calculated by iterative curve-fitting software (KaleidaGraph 3.0) using the Michaelis-Menten function, giving a $K_M$ of 41.5 ± 10.8 mM and a $J_{\text{max}}$ of 68.8 ± 7.0 mmol/s · L cell water.

**Effect of external dimethylamiloride concentration on $H^+$ efflux**

Amiloride and its derivatives have been shown to effectively block $Na^+/H^+$ exchange activity. Dimethylamiloride is a $Na^+/H^+$ exchange inhibitor with much greater efficacy and specificity (Vilella et al., '95) and severely retards $Na^+$-dependent $pH_i$ recovery in E-cells. In order to accurately describe the inhibitory kinetics of dimethylamiloride on the E-cell $Na^+/H^+$ exchange mechanism, an experiment was conducted with E-cells suspended in standard LPS with varying amounts of dimethylamiloride (0–50 µM). E-cells were acidified with 30 mM propionate and $H^+$ efflux measured over the first minute of recovery. Figure 4 is a Dixon transformation and illustrates the effect of increasing dimethylamiloride concentrations at external $Na^+$ concentrations of 50 mM and 400 mM. Single slopes for

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Fig. 3. $Na^+/H^+$ exchange kinetics of E-cells. External $Na^+$ concentrations ranged from 0 to 200 mM. Cells were acidified by exposing them to 30 mM K-propionate and rate of $H^+$ efflux was calculated over the first minute. Data plotted represent means ±S.E.M. of five calculations. Hyperbolic curve and kinetic constants were calculated using iterative curve-fitting software (KaleidaGraph 3.0).

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Fig. 4. Dixon plot of the effects of external dimethylamiloride (DMA). E-cells were exposed to a range of DMA (0–50 µM) while suspended in medium containing either 20 mM or 370 mM $Na^+$ and acidified with the addition of 30 mM Na-propionate. Rates of $H^+$ efflux were measured over the first minute of recovery. Values are means ± S.E.M. of three separate preparations. Lines were drawn by linear regression analysis.

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each Na⁺ concentration suggest a single binding site for dimethylamiloride on the Na⁺/H⁺ exchanger. The intersections of the plots at 50 mM and 400 mM are above the horizontal axis, indicating competitive binding of dimethylamiloride to the antiporter and a $K_i = 630$ nM.

**Effect of phorbol ester and 8-Br-cAMP on Na⁺/H⁺ exchange**

The resting pHᵢ of E-cells is $7.19 ± 0.008$ ($n = 12$) in standard LPS. Two chemical agents capable of stimulating either protein kinase A or protein kinase C pathways were applied to BCECF stained E-cell suspensions. E-cells were suspended in standard LPS, and test cells were incubated in variable concentrations (data not shown) of either phorbol 12-myristate 13-acetate (PMA) or 8-bromo-cyclic adenosine monophosphate (8-Br-cAMP). Addition of 20 nM PMA induced a considerable alkalinization over a 10-min period which became apparent 3 min after the addition of phorbol ester (Fig. 5). In contrast, 2 µM 8-Br-cAMP produced no such stimulation of Na⁺/H⁺ exchange over the entire 10-min interval. The final pHᵢ attained after the addition of 20 nM PMA for three separate experiments was $7.45 ± 0.02$, an average pHᵢ increase of 0.21.

**Effect of internal H⁺ on Na⁺/H⁺ exchange and mechanism for phorbol ester activation**

The possibility of an activating effect of internal H⁺ on Na⁺/H⁺ exchange activity was investigated by suspending BCECF-stained E-cells in a choline medium and incubating in nigericin (0.5 µg/mL) until a desired intracellular pH was obtained. After removal of nigericin (albumin 2.5 mg/mL) cells were resuspended in a LPS containing 70 mM Na⁺, and recovery to baseline pHᵢ levels was monitored. Proton efflux was calculated at each pHᵢ plotted on the abscissa in Figure 6 and is seen as the curve on the left. The data were curve-fitted according to the Hill equation:

$$J_H = J_{max}/[1+(10^{pH_i/10K})^n],$$

where $J_H$ is the outward H⁺ flux and $J_{max}$ is the maximal carrier-mediated H⁺ flux in µmol/s · L cell water, $pK$ is the pH at which H⁺ efflux is 1/2 of $J_{max}$, and n is the Hill coefficient for activation of the Na⁺/H⁺ exchanger by internal protons. The

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**Figure 5.** Effects of potential activating agents of E-cell Na⁺/H⁺ exchange. E-cells suspensions in a standard lobster physiological saline were exposed to 20 nM PMA (phorbol 12-myristate, 13-acetate) and 1 µM 8-Br-cAMP (8-bromo-cyclic adenosine monophosphate) in order to assess any activating effect. Over a period of 10 min, the presence of 20 nM PMA caused an increase of 0.28 pH units, whereas 8-Br-cAMP was without effect relative to control conditions. Data represent means ±S.E.M. of three separate preparations.

**Figure 6.** Effect of pHᵢ on rate of Na⁺/H⁺ exchange (circles). E-cells were acidified to specific pHᵢ values using the nigericin/albumin technique in a Na⁺-free physiological saline. Recovery to neutral pHᵢ was initiated with the addition of 70 mM Na⁺. H⁺ efflux was calculated during the first minute of recovery. Effect of phorbol ester on the internal H⁺ dependence of Na⁺/H⁺ activity (triangles). E-cell acidification and measurement of H⁺ efflux were performed as indicated in Figure 4. The presence of 10 nM PMA caused a shift in the sigmoidal curve towards more alkaline values essentially enabling the Na⁺/H⁺ exchanger to be activated at a higher pHᵢ than under control conditions. Data represent means ±S.E.M. of three preparations and the sigmoidal plot and kinetic values calculated using iterative curve-fitting software (KaleidaGraph 3.0).
kinetic constants were calculated from the curve-fitted data. Half-maximal activation was observed at pH 6.52 ± 0.05, maximal activity reached 0.58 ± 0.12 mmol/s · L cell water and the Hill coefficient was recorded as 3.2 ± 0.4 indicating a sigmoidal relationship between internal H⁺ and Na⁺/H⁺ exchange activity where as pH decreases, proton efflux increases.

**Effect of phorbol ester on intracellular H⁺ activation of Na⁺/H⁺ exchange**

Because it has been shown that phorbol ester activates Na⁺/H⁺ exchange in E-cells, experiments were conducted to ascertain any effect of PMA on the kinetic parameters of the Na⁺/H⁺ exchanger. E-cells were adjusted to variable pH as described above and preincubated for 10 min in 20 nM PMA. Following preincubation, E-cells were resuspended in LPS containing 70 mM Na⁺ and proton efflux measured. It is evident by examining the curve on the right in Figure 6 that phorbol ester treatment produced an alkaline shift of the whole pHi activation curve. Half-maximal activation was at a significantly higher pH than nontreated cells at 6.71 ± 0.03 (P < 0.05). Maximal activity on the other hand, remained unaffected and averaged 0.51 ± 0.03 µmol/s · L cell water. Further analysis of the data revealed that the allosteric activation of the antiporter also remained unaffected, the Hill coefficient averaged 2.94 ± 0.3. Application of phorbol ester produced one significant change in the kinetic parameters of lobster E-cell electroneutral exchangers: a pronounced alkaline shift in its activation curve.

**Effect of bisindoylmaleimide I on PMA-induced Na⁺/H⁺ exchange activation**

To determine whether the activation of Na⁺/H⁺ exchange by phorbol ester was mediated via a PKC pathway, the efficacy of a known potent inhibitor of PKC, bisindoylmaleimide I (BIM I), in preventing cellular alkalinization as a result of phorbol ester induced Na⁺/H⁺ exchanger was assessed. E-cells were prepared and suspended one of three solutions: LPS only (control), LPS + 20 nM PMA, or LPS + 20 nM PMA + 10 nM BIM I. The presence of 10 nM BIM I greatly reduced the intracellular rise in pH, that occurred in the presence of 20 nM PMA alone (Fig. 7). The final pH, attained by E-cells was 7.30 ± 0.02 in the presence of PMA and BIM I compared to 7.51 ± 0.01 (PMA) and 7.18 ± 0.02 (control).

**DISCUSSION**

The present study strongly supports the presence of a Na⁺/H⁺ exchanger on the plasma membrane of Atlantic lobster hepatopancreas E-cells. After an induced acid-load, purified E-cells displayed a Na⁺-dependent, dimethylamiloride-sensitive, recovery to a resting pH. Previous work has disclosed the presence of two types of Na⁺/H⁺ exchangers in hepatopancreas plasma membranes. An electrogenic 2Na⁺/1H⁺ exchanger (Ahearn and Clay, ’89) has been described for apical or brush-border membranes using purified vesicles (BBMV), whereas studies with basolateral membrane vesicles (BLMV) from the same tissue disclosed an electroneutral Na⁺/H⁺ exchanger (Duerr and Ahearn, ’96), functionally similar to members of the vertebrate NHE family of Na⁺/H⁺ exchangers (Yun et al., ’95). The data presented in this report suggest that lobster hepatopancreas E-cells possess only an electroneutral type of Na⁺/H⁺ exchange that is activated by intracellular H⁺ as well as by the phorbol ester PMA. The E-cell Na⁺/H⁺ exchanger is therefore physiologically very similar to Na⁺/H⁺ exchangers described for vertebrate species and may be functioning in E-cells in a highly conserved manner as a housekeeping protein, performing functions vital to cellular homeostasis such as intracellular pH and cell volume regulation.

Two lines of evidence suggest that the Na⁺/H⁺ exchanger expressed on the plasma membranes of E-cells is the electroneutral type described in basolateral membrane vesicle (BLMV) preparations. First, the presence of extracellular Li⁺ significantly inhibited (P < 0.05) Na⁺-dependent pHi recovery in E-cells after induced acid loading. Li⁺ competitively inhibits Na⁺/H⁺ exchange in BLMV with an apparent Ki = 493 µM (Duerr and Ahearn, ’96), whereas the BBMV Na⁺/H⁺ exchanger is unaffected by external Li⁺ (Z. Zhuang, unpubl. data). Second, the application of two monoclonal antibodies (MAb 9 and MAb11) that functionally inhibit the electrogenic 2Na⁺/1H⁺ exchanger in BBMV (DeCouet et al., ’93) have no inhibitory effect on Na⁺/H⁺ exchange in E-cell suspensions. To ensure effective antibody inhibition in whole-cell suspensions, the same experiment was conducted using a mixture of all four hepatopancreas cell types. In this case, a significant reduction in H⁺ efflux was measured (data not shown), indicating that a portion of Na⁺/H⁺ antiport in a mixed-cell population occurred by electrogenic 2Na⁺/1H⁺ exchange and that the monoclonal an-
antibodies could effectively inhibit such exchange in whole-cell preparations. Together these data suggest the sole presence of the electroneutral-type of Na⁺/H⁺ exchanger on E-cells.

The rate of H⁺ extrusion by the E-cell Na⁺/H⁺ exchanger was shown to be a hyperbolic function of extracellular Na⁺ concentration described by simple Michaelis-Menten kinetics as illustrated in Figure 3. Kinetic constants were determined using iterative curve-fitting software resulting in a calculated value for $K_{Na} = 41.5 \pm 10.8$ mM and a $J_{max}$ of $68.8 \pm 7.0 \mu$mol/s · L cell water. The $K_{Na}$ compares with that reported for the BLMV Na⁺/H⁺ exchanger of 28 ± 1.7 mM (Duerr and Ahearn, '96) and a range of 6 to 60 mM for vertebrate NHE isoforms (Clark and Limbird, '91). In contrast, the electrogenic 2Na⁺/1H⁺ exchanger present in BBMVs displays a sigmoidal relationship between Na⁺ uptake and external Na⁺ concentration with a $K_{Na}$ of 81 ± 7 mM and a Hill coefficient of 2.03 (Ahearn and Clay, '89).

In order to further demonstrate that this Na⁺-dependent pHᵢ recovery was mediated by a Na⁺/H⁺ exchanger, experiments delineating the effect of dimethylamiloride (DMA), a potent inhibitor of Na⁺/H⁺ exchange in vertebrate cells (Villella et al., '95), were conducted. A Dixon plot shown in Figure 4 illustrates the potent inhibitory effects of DMA on Na⁺-dependent H⁺ efflux in E-cells. DMA has a $K_i$ of 640 nM and acts competitively at a single binding site as revealed by single slopes for each Na⁺ concentration and their intersection above the x-axis. Reported $K_i$ values range from 40 nM (sensitive) to 20 μM (insensitive) (Clark and Limbird, '91), suggesting that the lobster Na⁺/H⁺ exchanger is relatively sensitive to DMA.

These kinetic data further support the notion that electroneutral Na⁺/H⁺ exchange occurs on the plasma membranes of E-cells and is the only type of Na⁺/H⁺ exchange present. E-cells are believed to be undifferentiated embryonic stem cells that
reside at the distal tip of hepatopancreas tubules that differentiate into the other cell types as they migrate proximally (Brunet et al., '94). The present results suggest that E-cells do not possess electrogenic 2Na⁺/1H⁺ exchange. The electrogenic antiporter that is only expressed on the apical membrane of certain differentiated hepatopancreas epithelial cells (Kimura et al., '94) may be a specialized transporter. In vertebrate epithelia, NHE isozymes coexist within the same cell but are restricted to opposite cell poles. NHE1, the ubiquitous Na⁺/H⁺ exchanger, exists on the plasma membrane of nearly all nonepithelial cells, but in polarized cells, it is restricted to the basolateral membrane. The apical membrane expresses all other NHE isoforms in a tissue-specific pattern. NHE1 is therefore assumed to play a housekeeping role. In lobster E-cells, it is conceivable that the electroneutral Na⁺/H⁺ exchanger represents a crustacean ortholog of the ubiquitous vertebrate NHE1 and is likewise functioning singly as a housekeeping antiporter in an undifferentiated epithelial cell.

Aronson et al. ('82) first proposed that intracellular H⁺ acts as an activator of Na⁺/H⁺ exchange after documenting that efflux of H⁺ via Na⁺/H⁺ exchange shows a steeper dependence on internal pH than would be expected for a simple Michaelis-Menten process in rat renal BBMVs. In view of considerable data supporting a stoichiometry of 1:1 for Na⁺/H⁺ exchange, it was proposed that intracellular H⁺ could bind and allosterically activate Na⁺/H⁺ exchange through an interaction that is independent of its role as a transportable substrate (Aronson et al., '82). This phenomenon was also observed in whole rat (Grinstein et al., '84) and human (Strazzullo and Canessa, '90) thymocytes. In contrast, lobster hepatopancreas electrogenic 2Na⁺/1H⁺ exchange displayed a hyperbolic relationship between Na⁺ uptake and pHᵢ, suggesting no activation effect of internal H⁺ (Ahearn and Clay, '89).

The relationship between pHᵢ and H⁺ efflux via the E-cell Na⁺/H⁺ exchanger was investigated in the present study. As illustrated in Figure 6, Na⁺-dependent H⁺ efflux was observed to be a sigmoidal function of pHᵢ in lobster E-cells described by the Hill equation (see equation 2 in Results). Hill plot analysis was performed using iterative curve-fitting software, which indicated a pK = 6.52 ± 0.05, Jₚₚₕₓ = 0.58 ± 0.12 μmol/s · L cell water, and a Hill coefficient (n) = 3.2 ± 0.4. Na⁺/H⁺ exchange activity was not detectable at pHᵢ > 7.0 indicating a set-point for a “pH-stat” in which Na⁺/H⁺ exchange activity increases as pHᵢ decreases below 7.0. These findings suggest that the lobster E-cell Na⁺/H⁺ exchanger is activated in an allosteric fashion by intracellular H⁺.

NHE1 has been separated into two functional domains. One is the transporter domain, which possesses all features necessary for transport as well as a built-in H⁺ binding modifier site. The other domain is a cytoplasmic regulatory domain, which mediates growth factor signals by interacting with the H⁺ sensor (Wakabayashi et al., '92). The activity of Na⁺/H⁺ exchangers can be modulated by a wide variety of stimuli, including growth factors (Moolenaar et al., '83; L'Allemand et al., '84), phorbol esters (Grinstein et al., '85), chronic extracellular acidification (Horrie et al., '90), and various signal transduction or second-messenger molecules (Bianchini and Pouyssegur, '94). NHE1 is activated by phorbol esters, compounds that mimic the actions diacylglycerol by activating PKC, and therefore it is believed that NHE1 is phosphorylated by Gq-mediated cell signalling (Grinstein et al., '85; Sardet et al., '90). However, NHEβ, an NHE1 homolog in telost red blood cells, is activated by cAMP (Borgese et al., '94) and epinephrine (Bernenbrink and Bridges, '94); cAMP has no effect on the activity of NHE1 (Bianchini and Pouyssegur, '94).

In order to assess whether the lobster E-cell Na⁺/H⁺ exchanger was physiologically more similar to NHE1 or NHEβ, the effects of phorbol ester and 8-Br-cAMP were assessed. The phorbol ester phorbol 12-myristate, 13-acetate (PMA) had a marked effect on resting pHᵢ of E-cell suspensions where 8-Br-cAMP had no effect at all (Fig. 5). The stimulatory effect could be blocked by DMA (data not shown), and this increase in pHᵢ was attributed to an increase in Na⁺/H⁺ exchange. The lobster E-cell Na⁺/H⁺ exchanger is thus more like NHE1 in its response to PMA and, potentially, PKC phosphorylation.

In order to address a mechanism of activation, the kinetic parameters of E-cell Na⁺/H⁺ exchangers were examined after incubation in PMA. Treatment of E-cells with 20 nM PMA for 10 min significantly (P < 0.05) increased the pK of the antiporter H⁺-binding site from 6.52 ± 0.05 to 6.71 ± 0.03. No significant change (P > 0.05) in the Hill coefficient or the Jₚₚₕₓ was measured. Therefore, activation of the E-cell Na⁺/H⁺ exchanger is brought about by a change in the cytoplasmic affinity of the antiporter for intracellular H⁺ and not by an increase in the number of antiporters residing in the plasma membrane.
Cell signalling pathways appear to be rather conserved across several phyla. Evidence shows that in barnacle muscle fibers, there is PKC (Bittar and Girard, '87) that is activated by phorbol esters. After fertilization, sea urchin eggs experience rapid ionic changes that are mediated by Na'/H+ exchange and which may be stimulated by phorbol ester activation of PKC (Ciapa et al., '89). Finally, in crayfish hepatopancreas, the mode of action of crustacean hyperglycemic hormone (CHH) is mediated by cyclic nucleotides and cyclic nucleotide-dependent protein kinases (Sedlmeyer, '85). Therefore it is not inconceivable that the E-cell Na+/H+ exchanger, as a conserved housekeeping protein (an NHE1 lobster ortholog), is regulated in a similarly conserved manner.

The role of phosphorylation in activation of Na+/H+ exchange has been documented by Sardet et al. ('90). After a lag of two minutes, the presence of phorbol ester induced an increase in pH, accompanied by phosphorylation of the antiporter at serine residues. Exposure of rabbit renal brush-border membrane vesicles to exogenous PKC increased the rate of proton-stimulated, amiloride-sensitive sodium transport (Weinman and Shenolikar, '86). Incubation of cultured renal epithelial cells in acid medium caused an increase in Na+/H+ antiporter activity that was prevented by the addition of a PKC inhibitor (Horie et al., '92). Parathyroid hormone has been shown to regulate Na+/H+ exchange via an interaction with both PKC and protein kinase A via a G protein coupled receptor (Azarani et al., '95). Furthermore, calyculin A (a protein phosphatase inhibitor) effectively blocks desensitization of the βNHE (Guizouarn et al., '95).

There is evidence to support the role of PKC in a regulatory model for E-cell Na+/H+ exchange activity. The presence of bisindoylmaleimide I, a potent PKC inhibitor, in lobster E-cells treated with a known PKC activator (20 nM PMA) greatly reduced an otherwise significant rise in pH, induced by the phorbol ester. This evidence strongly suggests a role for PKC in the regulation of lobster hepatopancreas E-cell electroneutral Na+/H+ exchange.

The present study constitutes the first investigation into the regulation of plasma membrane transporters in isolated crustacean cells and describes a sole electroneutral Na+/H+ exchanger residing on the plasma membrane of purified lobster hepatopancreas E-cells. This Na+/H+ exchanger physiologically resembles members of the vertebrate NHE family and is regulated in a manner similar to that described for NHE1. Recently, a cDNA clone of a Na+/H+ exchanger has been isolated from crab gill (Towle et al., '97) using RT-PCR. Both structural and functional evidence now exist to support the notion that electroneutral Na+/H+ exchange is highly conserved and is present in crustacean tissues.

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