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Characterization of Na^+/H^+ exchange in FRTL-5 thyroid cells. Evidence for dependence on activation of protein kinase C

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Na^+/H^+ exchange activity was investigated in cultured rat thyroid follicular FRTL-5 cells using the pH sensitive dye 2',7'-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF). Basal intracellular pH (pH_i) was 7.13 ± 0.10 in cells incubated in Hepes-buffered saline solution. The intracellular buffering capacity β_i was determined using the NH_4Cl-pulse method, yielding a β_i value of 85 ± 12 mM/pH unit. The relationship between extracellular Na^+ and the initial rate of alkalinization of acid-loaded cells showed simple saturation kinetics, with an apparent K_m value of 44 ± 26 mM, and an V_{max} value of 0.3 ± 0.01 pH unit/min. The agonist-induced activation of Na^+/H^+ exchange was investigated in cells acidified with nigericin. Addition of 12-*O*-tetradecanoylphorbol 13-acetate (TPA) or ATP induced rapid cytosolic alkalinization in acid-loaded cells. The action of both TPA and ATP was abolished by preincubating the cells with 100 μM amiloride, by substituting extracellular Na^+ with equimolar concentrations of choline$^+$, and by pretreating the cells with TPA for 24 h. Chelating extracellular Ca^{2+}, or depleating intracellular Ca^{2+} pools did not affect the ATP-induced alkalinization. The results indicate, that FRTL-5 cells have a functional Na^+/H^+ exchange mechanism. Furthermore, stimulation of protein kinase C activity is of importance in activating the antiport.

Introduction

It is generally considered, that the regulation of intracellular pH (pH_i) is of crucial importance in regulating several cell functions, including cell growth and differentiation [1], and modification of release of intracellular sequestered Ca^{2+} [2,3]. One important regulator of pH_i is the Na^+/H^+ exchange mechanism. Na^+/H^+ exchange can be activated via several pathways: activation of protein kinase C (PKC) [1], an increase in intracellular free Ca^{2+} [4–6], via activation of calmodulin [7,8], or an increase in intracellular cAMP [9].

In thyroid cells, the transport of iodide (I^-) is of central importance. Previous studies have shown, that I^- is cotransported with Na^+ into the cells [10]. This transport is dependent on the Na^+ gradient over the cell membrane, and on extracellular pH. Furthermore, in a recent report, accumulation of intracellular I^- was associated with a rapid acidification of pH_i, suggesting a relationship between regulation of pH_i and uptake of I^- in FRTL-5 cells [11]. However, no characterization of the Na^+/H^+ exchange was made. In the present study in FRTL-5 cells, we have investigated the importance of Na^+/H^+ exchange in the regulation of pH_i in FRTL-5 cells. In addition, we have investigated the possible mechanism of activation of the antiporter. Our results show, that stimulation of protein kinase C is an important pathway in the activation of the Na^+/H^+ exchange in FRTL-5 cells.

A preliminary report on these findings has been presented [12].

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Materials and Methods

Materials

Culture medium, serum and hormones needed for the cell culture were purchased from Gibco (Grand Island, NY, USA) and Sigma (St. Louis, MO, USA). Culture dishes were obtained from Falcon Plastics (Oxnard, CA, USA). Nigericin, monensin, TPA, and ATP were purchased from Sigma. Bis(carboxyethyl)carboxyfluorescein acethoxymethyl ester (BCECF-AM) was purchased from Molecular Probes, Inc. (Eugene, OR, USA), and ionomycin from Calbiochem (San Diego, CA, USA). All other chemicals used were of reagent grade.

Cell culture

Rat thyroid FRTL-5 cells were a generous gift of Dr. Leonard D. Kohn (NIH, Bethesda, MD). The cells were grown in Coon's modified Ham's F 12 medium, supplemented with 5% calf serum and six hormones [13] (6H: insulin, 10 μg/ml; transferrin, 5 μg/ml; hydrocortisone, 10 nM; the tripeptide Gly-L-His-L-Lys, 10 ng/ml; TSH, 1 mU/ml; somatostatin, 10 ng/ml). In some experiments, TSH was omitted from the medium (5H). The cells were grown in a water-saturated atmosphere of 5% CO_2 and 95% air at 37°C. Before an experiment, cells from one donor culture flask were harvested with a 0.25% trypsin solution, and plated onto 100-mm culture dishes. The cells were grown for 7–8 days before an experiment, with two or three changes of the culture medium. Fresh medium was always added 24 h prior to an experiment.

Measurement of pH_i

The method for measuring pH_i in FRTL-5 cells was essentially similar to that previously described for GH_4C_1 cells [6,14]. In brief, the cells were harvested with Hepes-buffered salt solution (HBSS; containing in millimolar concentrations: NaCl, 118; KCl, 4.6; glucose, 10; $CaCl_2$, 1.0; Hepes, 20.0; pH 7.2) without $CaCl_2$, but containing 0.02% EDTA and 0.01% trypsin, washed twice in $CaCl_2$-containing HBBS, and incubated for 35 min at 37°C with 5 μM of BCECF-AM. pH_i was determined fluorometrically with a Hitachi F2000 fluorometer, using an excitation wavelength of 500 nm, and an emission wavelength of 530 nm. The dye leakage from the BCECF-loaded cells was less than 10% in 30 min. At the end of the experiment, the signal was calibrated by lysing the cells with digitonin and measuring the fluorescence of known pH values. To correct for the red shift in the spectrum of BCECF induced by calibrating BCECF in an extracellular solution, cells were incubated in a high-K^+ buffer, and known pH values were imposed inside the cells by using 10 μg/ml of the K^+/H^+ ionophore nigericin. The cells were then lysed with digitonin and a new calibration curve constructed [15]. The calibration curves were linear over the pH range between 6.4 and 7.2.

Buffering capacity

The intracellular buffering capacity β_i was determined from the changes in pH_i induced by challenging the cells with NH_4^+/NH_3 using 20 mM NH_4Cl [16]. β_i was calculated from the formula

$$\beta_i = \Delta NH_{4i}^+ / \Delta pH_i$$

where ΔNH_{4i}^+ is the amount of intracellular NH_4^+ formed, and ΔpH_i is the measured change in pH_i.

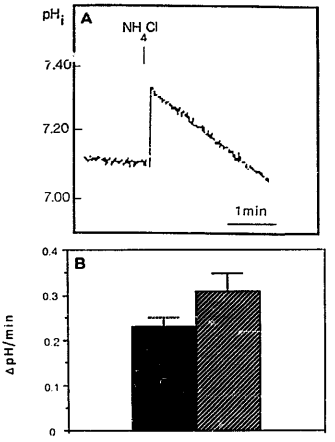

Fig. 1. Intracellular buffer capacity in FRTL-5 cells. (A). The cells were challenged with 20 mM NH_4Cl at basal pH_i. (B). Comparison of the change in pH_i in response to 20 mM NH_4Cl at basal pH_i (■), or after acid-loading the cells to 6.9 ± 0.03, using a 40 mM NH_4Cl prepulse (▨).

Statistics

The results are expressed as the mean ± S.E. The ΔpH_i was calculated as the change in pH_i ($pH_{(after\ addition)} - pH_{(before\ addition)}$) during 1 min after addition of agonist. The traces shown are representative traces from each group of experiments. Statistical analysis was made using Student's *t*-test for paired observations.

Results

Intracellular buffer capacity β_i

To determine β_i, the cells were challenged with 20 mM NH_4Cl (Fig. 1). The calculated value for β_i was 84 ± 7 mM/pH unit. In cells acidified to 6.9 ± 0.03 with 40 mM NH_4Cl using the pre-pulse method [17], the calculated β_i value was 83 ± 14 mM/pH unit (Fig. 1).

Manipulation of pH_i

The resting pH_i in FRTL-5 cells was 7.13 ± 0.10 ($n = 63$). Addition of 100 μM amiloride decreased basal pH_i 0.12 ± 0.02 unit. Furthermore, substituting extracellular Na^+ with equimolar concentrations of $choline^+$ slowly lowered pH_i to 6.89 ± 0.21 ($P < 0.05$).

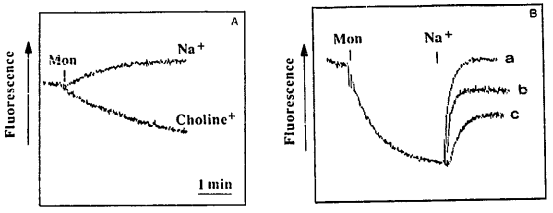

Fig. 2. Effect of monensin on pH_i in FRTL-5 cells. (A). Stimulating the cells with 1 μM monensin in Na^+ buffer, or choline$^+$ buffer. (B). Addition of 80 (a), 40 (b), or 20 (c) mM Na^+ to cells acidified with 10 μM monensin in choline$^+$ buffer.

To demonstrate that removal of extracellular Na^+ reversed the Na^+-gradient of the cells, the electroneutral Na^+/H^+ ionophore monensin was tested (Fig. 2A). Addition of 10 μM monensin to cells suspended in choline$^+$ buffer decreased pH_i 0.42 $\pm$ 0.02 unit (Fig. 2B). Addition of Na^+ rapidly restored pH_i to basal values.

Dependence of Na^+/H^+ exchange on extracellular Na^+

To examine the relationship between extracellular Na^+ and the activation of the Na^+/H^+ exchange in FRTL-5 cells, the cells were acid-loaded by preincubation with 20 mM NH_4Cl for 10 min, decreasing pH_i to 6.50 $\pm$ 0.01. Addition of Na^+ to the cells induced a dose-dependent increase in the rate of alkalinization (Fig. 3A). The rate of alkalinization increased according to simple Michaelis-Menten saturation kinetics. A Lineweaver-Burke plot of the data yielded an apparent K_d value of 44 $\pm$ 26 mM ($n = 5$) for Na^+ and a V_{max} value of 0.3 $\pm$ 0.10 pH unit/min (Fig. 3B). Amiloride inhibited in a dose-dependent manner the alkalinization induced by addition of 80 mM Na^+ to acid-loaded cells (Fig. 4).

Activation of Na^+/H^+ exchange in FRTL-5 cells

Na^+/H^+ exchange may be activated via several pathways, including stimulation of PKC [1], an increase in $[Ca^{2+}]_i$ [6], and stimulation of cAMP production [9]. We could not detect any changes in basal pH_i when FRTL-5 cells were stimulated with 200 nM of the phorbol ester TPA, 30 μM ATP, 100 μM NaI, or 10 mU/ml TSH (not shown). In addition, TSH had no effect on basal pH_i in cells incubated in 5H for three days (not shown). To further study the activation of the Na^+/H^+ exchange mechanism, it was thus necessary to increase the intracellular acid load of the cells using the K^+/H^+ ionophore nigericin [6,18]. Addition of nigericin (1 μg/ml) rapidly decreased pH_i by 0.5–0.6 pH unit (Fig. 5). Addition of 10 mU TSH or 100 μM NaI had no effect on pH_i in acid-loaded cells (not shown). However, addition of 200 nM TPA to these cells caused a rise in pH_i of 0.03 $\pm$ 0.01 pH unit ($n = 7$) within 1 min (Fig. 5B). This increase was significant ($P < 0.05$) compared with vehicle-treated cells (0.00 $\pm$ 0.01, $n = 4$; Fig. 5A). To determine whether the in-

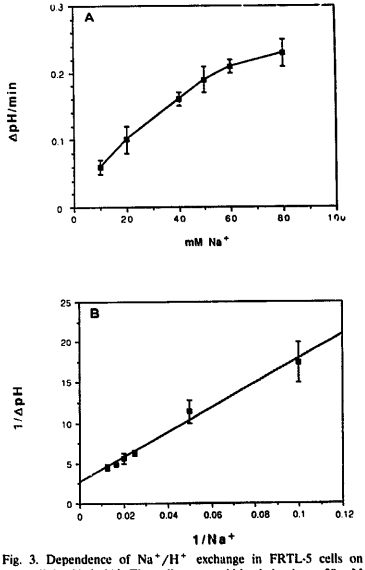

Fig. 3. Dependence of Na^+/H^+ exchange in FRTL-5 cells on extracellular Na^+. (A). The cells were acid-loaded using a 20 mM $NHCl_4$ prepulse in choline$^+$ medium. Addition of extracellular Na^+ induced a dose-dependent alkalinization. (B). Lineweaver-Burke plot of the data in A. Each point gives the mean $\pm$ S.E of five independent experiments.

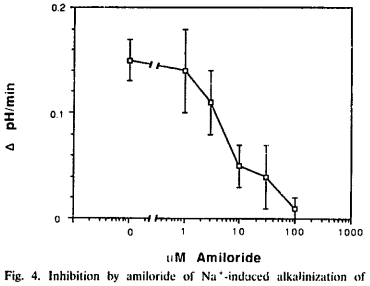

Fig. 4. Inhibition by amiloride of Na^+-induced alkalinization of acid-loaded FRTL-5 cells.The cells were acid-loaded using a 40 mM $NHCl_4$ prepulse in choline$^+$ medium. The cells were then incubated with increasing doses of amiloride for 2–3 min, and 80 mM Na^+ was added. Each point gives the mean ± S.E. of five determinations.

crease in pH_i induced by TPA was dependent on activation of Na^+/H^+ exchange, the effect of amiloride and Na^+-free buffer was tested. Preincubating the cells with 100 μM amiloride for 3–5 min totally inhibited the TPA-induced alkalinization (Fig. 5C). Substituting extracellular Na^+ with equimolar concentrations of choline$^+$ abolished the TPA-induced alkalinization (Fig. 5D). Furthermore, in cells where PKC had been down-regulated by incubating the cells with 200 nM TPA for 24 h [19], addition of TPA had no effect on pH_i (not shown). The results indicate that, in FRTL-5 cells, Na^+/H^+ exchange can be activated via stimulation of PKC.

We then investigated whether changes in intracellular free Ca^{2+} ($[Ca^{2+}]_i$) could activate Na^+/H^+ exchange in FRTL-5 cells, using the purinergic agonist ATP. ATP has been shown to potently stimulate an increase in $[Ca^{2+}]_i$ both via release on intracellular sequestered Ca^{2+}, and influx of extracellular Ca^{2+} [20,21]. In Fig. 6B we show, that stimulating acid-loaded cells with 30 μM ATP increased pH_i by 0.04 ± 0.01 pH unit ($n = 5$, $P < 0.05$ as compared with 0.00 ± 0.01 pH unit in control cells, $n = 4$). The ATP-induced increase in pH_i was mediated via activation of Na^+/H^+ exchange, as the response was inhibited by preincubating the cells with 100 μM amiloride (Fig. 6C), and by substituting extracellular Na^+ with equimolar concentrations of choline$^+$ (Fig. 6D). The effect of ATP was not dependent on extracellular Ca^{2+}, as ATP increased pH_i to the same extent in both a Ca^{2+}-containing buffer (0.04 ± 0.01, $n = 5$), and a Ca^{2+}-free buffer (0.04 ± 0.01, $n = 5$). If intracellular Ca^{2+} stores were depleated with the Ca^{2+} ionophore ionomycin, the increase in pH_i in response to ATP was 0.03 ± 0.01 pH unit ($n = 5$) compared with 0.00 ± 0.01 in control cells ($n = 4$, $P < 0.05$). However, if the cells were pretreated

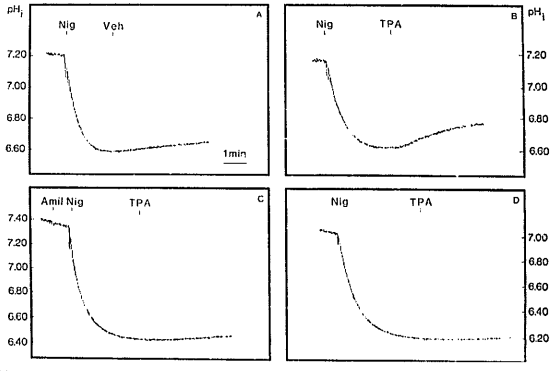

Fig. 5. Effect of TPA on pH_i in FRTL-5 cells acid-loaded by pretreatment with nigericin. Nigericin (Nig; final concentration 1 μg/ml) was added. (A). Addition of DMSO (vehicle, Veh). (B). Addition of 200 nM TPA. (C). Addition of 100 μM amiloride (Amil) prior to addition of nigericin and TPA. (D). Addition of 200 nM TPA to cell suspended in a buffer were Na^+ was substituted with equimolar concentrations of choline$^+$.

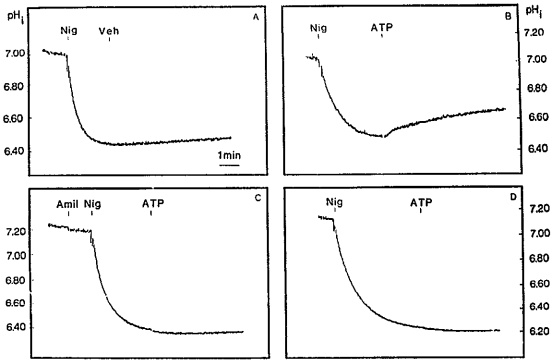

Fig. 6. Effect of ATP on pH_i in FRTL-5 cells acid-loaded by pretreatment with nigericin. Nigericin (Nig; final concentration 1 μg/ml) was added. (A) Addition of vehicle (Veh). (B) Addition of 30 μM ATP. (C). Addition of 100 μM amiloride (Amil) prior to addition of nigericin and ATP. (D). Addition of 30 μM ATP to cell suspended in a buffer were Na^+ was substituted with equimolar concentrations of choline$^+$.

with 200 nM TPA for 24 h, thus down-regulating PKC, no significant increase in pH_i was observed in response to ATP (0.03 ± 0.02 pH unit ($n = 5$) compared with 0.03 ± 0.01 pH unit in control cells ($n = 4$). The results thus suggest, that ATP stimulates Na^+/H^+ exchange in FRTL-5 cells via activation of PKC.

Discussion

In the present report, we have characterized the basic kinetic properties of the Na^+/H^+ exchange mechanism in FRTL-5 cells. Our results show, that the K_m value, the V_{max} value, and the intracellular buffering capacity β_i for the Na^+/H^+ exchange mechanism are comparable to those observed in other cell systems [16,22]. In addition, we have shown that stimulation of PKC-activity is an important signal in activation of the antiport. Our results regarding the importance of activation of PKC are in agreement with the results reported by Woods et al. [23], although we could not observe an effect of TPA on basal pH_i.

The importance of PKC in regulating Na^+/H^+ exchange has previously been shown in several cell systems, although other pathways have also been shown to exist, such as osmotic shrinkage, activation of tyrosine kinase activity, or increase in $[Ca^{2+}]_i$ (see Ref. 1 for a recent review). The mechanism of action of PKC on the Na^+/H^+ exchange molecule is still not totally understood, although Sardet et al. [24] have shown that the time course for the epidermal growth factor (EGF)-induced alkalinization of hamster fibroblasts and human epidermoid A431 cells is identical with the phosphorylation of the Na^+/H^+ exchange molecule.

In a recent report, Marcocci and Grollman [11] observed a clear decrease in pH_i in FRTL-5 cells after addition of I^-. We did not observe any decrease. The reason for the discrepancy in our results is unknown. As I^- is cotransported with Na^+ into the cells, uptake of I^- should be accompanied by an increase in intracellular Na^+ [10]. The increased intracellular concentration of Na^+ could theoretically compete with H^+ for the binding sites on the antiport, thus acidifying the cytosol [25]. It may be possible though, that the Na^+ cotransported with I^- is rapidly transported out of the cell by the Na^+/K^+-ATPase [10], making an interaction with the Na^+/H^+ exchange less likely.

TSH is considered to be of necessary for inducing proliferation in FRTL-5 cells [13,26,27]. TSH is a potent stimulator of cAMP formation, and cAMP has been shown to activate Na^+/H^+ exchange in murine macrophages [9]. Furthermore, an alkalinization of the cytosol is usually considered to be needed for proliferation to occur [1]. However, we could not obtain an activation of Na^+/H^+ exchange in response to TSH, irrespective of the experimental design. Similar results havebeen reported by Woods et al. [23]. In addition, physiological concentrations of TSH have been shown to activate the formation of the endogenous PKC-activator, 1,2-diacylglycerol (1,2-DG), suggesting that activation of PKC may be of importance in stimulating proliferation in FRTL-5 cells [28,29]. Furthermore, TPA has been shown to be an activator of cell prolifer-

ation in several cell systems [1], including thyroid cells [30]. This effect of TPA has generally been attributed to the TPA-induced activation of Na^+/H^+ exchange [1]. It is thus theoretically possible, that both TPA and TSH could induce proliferation through the same mechanism, i.e. activation of PKC. An other stimulator of proliferation in FRTL-5 cells, insulin-like growth factor-1 (IGF-1), has been shown to increase 1,2-DG [28], and to be a potent activator of Na^+/H^+ exchange [23,31]. However, our results suggest, that the TSH-induced proliferation in FRTL-5 cells is probably not mediated via a rapid activation of Na^+/H^+ exchange.

The effect of the purinergic P_2 receptor agonist ATP is mediated via hydrolysis of phosphatidyl 4,5-bisphosphate to inositol-1,4,5-trisphosphate (IP_3) and 1,2-DG [32,33]. IP_3 is generally considered to release intracellular sequestered Ca^{2+} [34]. ATP has recently been shown to release sequestered Ca^{2+} and to induced influx of extracellular Ca^{2+} in FRTL-5 cells [20,21,34]. Agonist-induced increases in $[Ca^{2+}]_i$ has been shown to stimulate Na^+/H^+ exchange in several cell systems, including fibroblasts and pituitary cells [4–6], and influx of extracellular Ca^{2+} enhances the activity of Na^+/H^+ exchange in T3T cells [35]. In addition, ATP has been shown to activate Na^+/H^+ in endothelial cells via both a change in $[Ca^{2+}]_i$ and activation of PKC [36]. However, the ATP-induced increase in $[Ca^{2+}]_i$ had no effect on pH_i in FRTL-5 cells, and ATP-induced changes in $[Ca^{2+}]_i$ were not necessary for activation of Na^+/H^+ exchange. The results further support our suggestion, that stimulation of Na^+/H^+ exchange in FRTL-5 cells is mediated via activation of PKC.

Acknowledgements

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