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Characterization of the TRH-induced activation of Na^+/H^+-exchange in pituitary GH_4C_1 cells

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In the present study in GH_4C_1 cells, the dependence of TRH-induced activation of Na^+/H^+-exchange on extracellular Na^+ and Ca^{2+} was examined. Furthermore, the effects of both extracellular and intracellular H^+ on Na^+/H^+-exchange were investigated. The buffering capacity was 63 ± 11.8 mM (pH unit)$^{-1}$ at basal intracellular pH (pH_i) of 7.02 ± 0.02. The initial rate of alkalinization in cells acidified with nigericin increased with increasing concentrations of extracellular Na^+ according to simple Michaelis-Menten kinetics. The apparent K_m-value for Na^+ was 53 ± 17.5 mM and the V_{max} value was 28 ± 4.5 mM H^+/min. Addition of Na^+ together with TRH increased V_{max} to 56 ± 6.4 mM H^+/min ($P < 0.05$), while no difference was observed in K_m. Decreasing extracellular pH (pH_o) decreased the rate of alkalinization of acid-loaded cells, despite a large inward Na^+ gradient. Furthermore, a decrease in pH_i was necessary to obtain activation of Na^+/H^+ exchange. At pH_i-values close to basal pH_i no activation of Na^+/H^+-exchange was obtained. In addition, the results showed that extracellular Ca^{2+} was necessary for TRH-induced activation of Na^+/H^+ exchange. Blocking influx of extracellular Ca^{2+} with Ni^{2+} abolished the effect of TRH, suggesting that the TRH-induced activation of Na^+/H^+-exchange in GH_4C_1 cells is dependent on influx of extracellular Ca^{2+}.

Introduction

In most cell types, including GH_4C_1 cells [1,2], intracellular pH (PH_i) is maintained in part by an electroneutral membrane-associated Na^+/H^+-exchange mechanism (see Ref. 3). Activation of Na^+/H^+-exchange has been shown to occur in response to osmotic shrinkage, an increase in intracellular free Ca^{2+} ($[Ca^{2+}]_i$), or activation of protein kinase C (PKC) (see Ref. 3). A multitude of cellular processes appears to be dependent on pH_i. Changes in pH_i are probably important in inducing proliferation and differentiation and in regulating cell volume [4]. Recent studies also suggest, that changes in pH_i may have a regulatory function on Ca^{2+} sequestration and release [5–7].

Stimulating GH_4C_1 cells with TRH rapidly hydrolyses phosphatidylinositol-4,5-bisphosphate to inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol [8,9]. IP_3 has been shown to release sequestered intracellular Ca^{2+} [10,11], and diacylglycerol to activate protein kinase C (PKC) [12]. Both an increase in intracellular Ca^{2+} ($[Ca^{2+}]_i$) and activation of PKC are important activators of Na^+/H^+-exchange in GH_4C_1 cells [1,2]. Although several reports on the regulation of pH_i in GH_4C_1 cells have been published [1,2,13,14], no characterization of the basic properties of the antiport have been made. This should be of importance, considering the apparently complex interaction between $[Ca^{2+}]_i$ and pH_i in GH_4C_1 cells [2,15]. Furthermore, knowledge of the basic kinetic behaviour of the exchanger is important in understanding the mechanism of activation of the antiport. Thus, in the present study, the kinetic properties of the TRH-induced activation of Na^+/H^+-exchange were determined. Furthermore, the results show that TRH-induced influx of extracellular Ca^{2+} is of importance in activating Na^+/H^+-exchange in GH_4C_1 cells.

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Materials and Methods

Materials. BCECF-AM was obtained from Molecular Probes (Eugene, OR, USA). TRH, digitonin and nigericin were from Sigma (St Louis, MO, USA). All flasks and dishes used for the cell culture were from Nunc Plastics (Kamstrup, Denmark).

Cell culture. Clonal rat pituitary GH_4C_1 cells were grown in monolayer culture in Ham's F 10 Nutrient Mixture with 15% (v/v) horse serum and 2.5% fetal bovine serum in a water-saturated atmosphere of 5%

CO_2 and 95% air at 37°C, as described previously [16,17]. Before an experiment, the cells from a single donor culture were harvested with 0.1% trypsin and subcultured in 100-mm culture dishes for 7–9 days. The cells were fed every 2–3 days and always the day before an experiment.

Measurement of pH_i. The method for measuring pH_i in GH_4C_1 cells has been described [1]. In brief, the cells were harvested in Hepes-buffered salt solution (HBSS, containing in millimolar concentrations: NaCl, 118; KCl, 4.6; glucose, 10; $CaCl_2$, 0.4; Hepes 20.0 (pH 7.2)) lacking $CaCl_2$ and containing 0.02% EDTA. The cells were washed twice in Ca^{2+}-containing HBBS and incubated for 35 min at 37°C with 5 μM bis(carboxyethyl)-carboxyfluorescein-AM (BCECF-AM). pH_i was determined fluorometrically with a Hitachi F2000 fluorometer (Hitachi, Tokyo, Japan) using an excitation wavelength of 500 nm and an emission wavelength of 530 nm. At the end of the experiment, the signal was calibrated by lysing the cells with digitonin and measuring the fluorescence at 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 across the plasma membrane using the K^+/H^+ ionophore nigericin. The cells were then lysed with digitonin and a new calibration curve constructed [18]. 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 [19]. β_i was calculated from the formula

$$\beta_i = \Delta(NH_4^+)_i/\Delta pH_i$$

where $\Delta(NH_4^+)_i$ is the amount of intracellular NH_4 formed and ΔpH_i is the measured change in pH_i. For calculation of $\Delta(NH_4^+)_i$, $(NH_3)_i$ was assumed to equal $(NH_3)_o$ and pK_a 9.24 was used. The acid extrusion rate (J_{H^+}) was calculated as the product of the rate of pH_i recovery and the buffering capacity [20].

Statistics. The results are expressed as the mean ± S.E. Each experiment was repeated at least four times, with at least three different batches of cells. Statistical analysis was made using Student's *t*-test for paired observations. Three or more means were tested using analysis of variance.

Results

Intracellular buffering capacity β_i of GH-cells

Addition of 20 mM NH_4Cl induced a rapid alkalinization in pH_i, due to influx of NH_3 (not shown). This initial alkalinization then declined towards basal levels, probably due to influx of NH_4^+ [21]. In the present report, the initial increase in pH_i was used for calculating β_i. At the basal pH_i-value of 7.02 ± 0.02, β_i was calculated to be 63 ± 11.8 mM (pH unit)$^{-1}$ (Fig. 1, $n = 4$), a value comparable with results obtained in other cell types [19]. The dependence of β_i on pH_i was investigated in cells acidified by addition of nigericin (1 μg/ml final concentration). The dependence of β_i on pH_i is shown in Fig. 1.

Kinetics of Na^+/H^+-exchange in GH_4C_1 cells

Previous studies have shown, that GH_4C_1 cells have a functional Na^+/H^+-exchange mechanism [1,2,13]. However, the kinetic properties of this exchange have not been determined. Addition of Na^+ to cells acidified by preincubation with nigericin [22] rapidly increased pH_i. The effect of Na^+ was dose-dependent, showing simple Michaelis-Menten kinetics (Fig. 2A). Addition of TRH together with Na^+ to the cell suspension enhanced the alkalinization. The Lineweaver-Burk plot of the data is shown in Fig. 2B. The values for Na^+ only and Na^+ together with TRH both fit a straight line. The apparent K_m-value was 53 ± 17.5 mM ($n = 5$) for Na^+ and 71 ± 8.8 mM ($n = 5$) for Na^+ together with TRH. The V_{max}-value for Na^+ was 28 ± 4.5 mM H^+/min and for Na^+ together with TRH 56 ± 6.4 mM H^+/min ($P < 0.05$).

Inhibitory effect of amiloride on Na^+/H^+ exchange in GH_4C_1 cells

Incubating the cells with 100 μM amiloride slowly lowered basal pH_i, indicating that some Na^+/H^+-exchange activity was present in unstimulated cells (data not shown). Activation of Na^+/H^+-exchange in cells acidified with nigericin was inhibited in a dose-dependent manner by amiloride (Fig. 3). The IC_{50}-value for

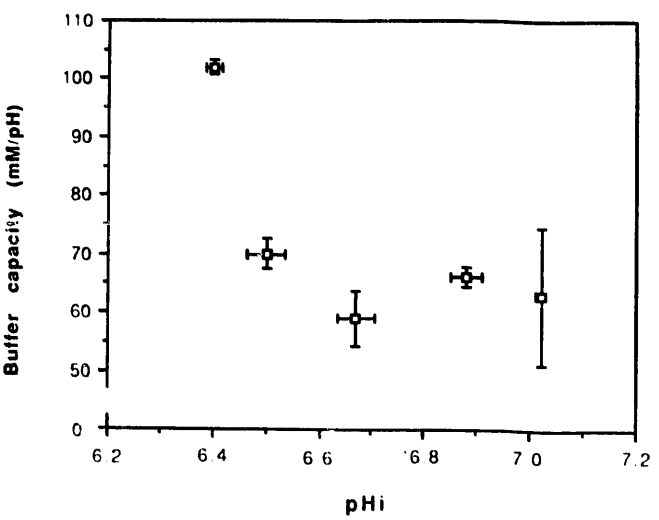
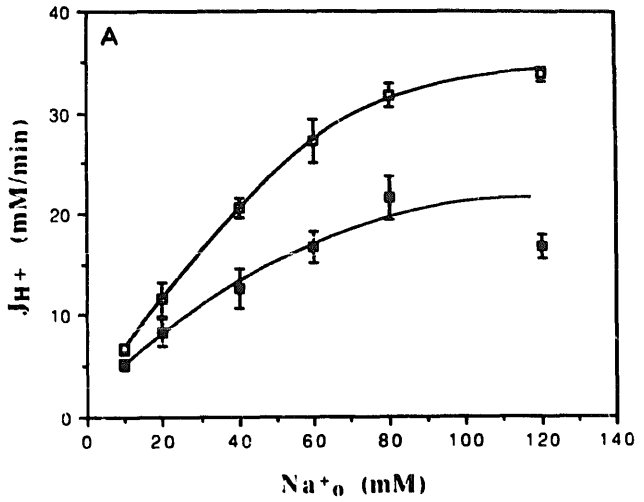


Fig. 1. Intracellular buffering capacity β_i in GH_4C_1 cells. BCECF-loaded cells were challenged with 20 mM NH_4Cl at basal pH_i (7.0), or after acidification with nigericin (final concentration 1 μg/ml). Each value is the mean ± S.E. of 4–6 determinations.



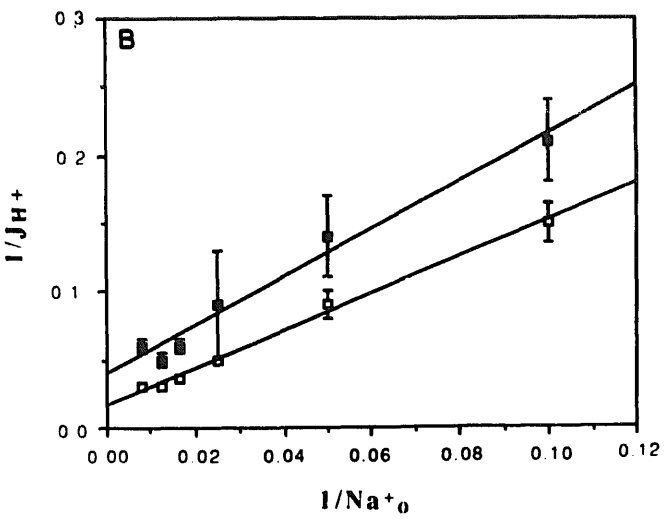


Fig. 2. Dependence of Na^+/H^+-exchange on extracellular Na^+ in GH_4C_1 cells. BCECF-loaded cells were acidified with nigericin (final concentration 1 μg/ml), washed and resuspended in choline$^+$ buffer. (A) Dose-dependent effect of Na^+ (■) and Na^+ together with 100 nM TRH (□) on pH_i. (B) Lineweaver-Burk plot of the data in A. Each point is the mean ± S.E. of five or six experiments.

inhibiting both the Na^+-induced alkalinization and the alkalinization obtained with Na^+ together with TRH was approx. 10 μM.

Dependence of TRH-induced activation of Na^+/H^+-exchange on extracellular and intracellular pH in GH_4C_1 cells

Several studies have indicated that H^+ might compete with Na^+ for binding to the extracellular binding site on the antiporter [23,24]. Fig. 4A shows the effect of extracellular pH (pH_o) on TRH-induced activation of Na^+/H^+-exchange in cells acidified with nigericin to 6.4 ± 0.05. At low pH_o, close to pH_i, Na^+/H^+-exchange was almost totally inhibited, although a large inward Na^+ gradient was present. The results in Fig.

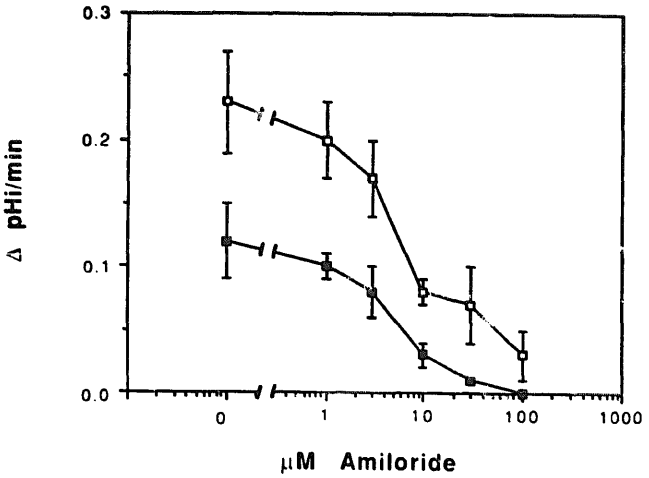


Fig. 3. Inhibitory effect of amiloride on Na^+/H^+-exchange in GH_4C_1 cells. BCECF-loaded cells were acidified as described in Fig. 2. The cells were incubated with the appropriate concentration of amiloride for 1 min and 80 mM Na^+ (■), or 80 mM Na^+ together with 100 nM TRH (□) was added and the change in pH_i was measured. Each point is the mean ± S.E. of 4–6 determinations.

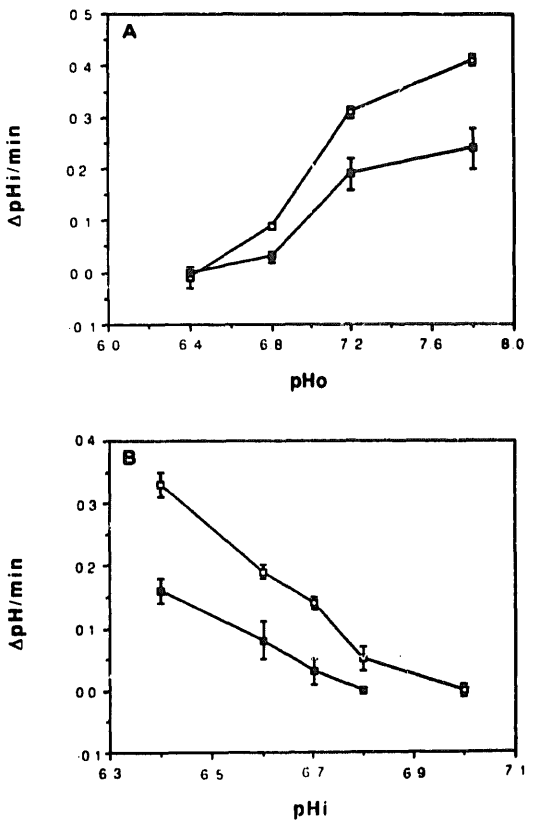


Fig. 4. Dependence of Na^+/H^+-exchange activity in GH_4C_1 cells on extracellular and intracellular pH. BCECF-loaded cells were acidified as described in Fig. 2. (A) The cells were resuspended in choline$^+$ buffer at the appropriate extracellular pH (pH_o). Then 80 mM Na^+ (■), or 80 mM Na^+ together with 100 nM TRH (□) was added and the change in pH_i was measured. (B) The cells were acidified as described in Fig. 2 to different pH_i-values. Then 80 mM Na^+ (■), or 80 mM Na^+ together with 100 nM TRH (□) was added and the change in pH_i was measured. Each point is the mean ± S.E. of 4–7 determinations.

4B show the dependence of TRH-induced activation of Na^+/H^+-exchange on pH_i. At values close to basal pH_i, no activation of the exchange can be detected.

Effect of extracellular Ca^{2+} and Ni^{2+} on TRH-induced activation of Na^+/H^+-exchange in GH_4C_1 cells

In a previous report, we showed that the TRH-induced activation of Na^+/H^+ exchange was decreased in a Ca^{2+}-free buffer [2]. The blunting effect of Ca^{2+}-free buffer on TRH-induced activation of Na^+/H^+-exchange is shown in Fig. 5B (compare to control in Fig. 5A). Furthermore, addition of 1 mM Ni^{2+} totally abolished the TRH-induced activation of Na^+/H^+-exchange (Fig. 5C). The effect of the vehicle is shown in Fig. 5D. The dose-dependent effects of extracellular Ca^{2+} and Ni^{2+} on TRH-induced activation of Na^+/H^+-exchange are shown in Fig. 6.

To test, whether Ca^{2+} per se could activate Na^+/H^+-exchange, 10 mM Ca^{2+} was added to acidified cells. This addition had no effect on Na^+/H^+-exchange (data not shown). Furthermore, addition of the dihydropyridine Ca^{2+} antagonist nimodipine did not inhibit TRH-induced activation of Na^+/H^+-exchange in acidified cells (data not shown). Taken together, the data indicate that influx of extracellular Ca^{2+} through a non-dihydropyridine Ca^{2+}-channel is necessary for the TRH-induced activation of Na^+/H^+-exchange in GH_4C_1 cells.

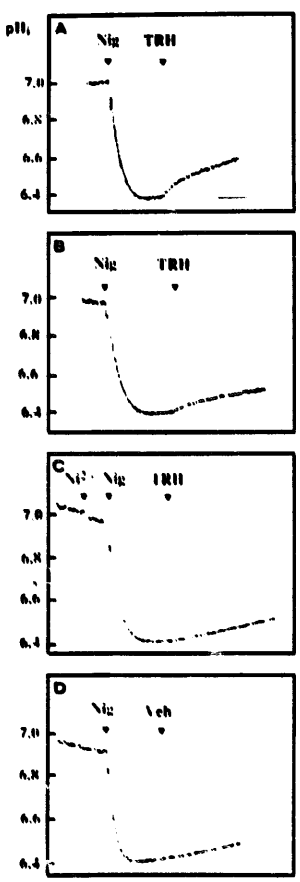


Fig. 5. Effect of extracellular Ca^{2+} on TRH-induced activation of Na^+/H^+-exchange in GH_4C_1 cells. The cells were acidified with nigericin (final concentration 1 μg/ml) as previously described [2]. Addition of 100 nM TRH to cells in: (A) Ca^{2+}-containing buffer; (B) Ca^{2+}-free buffer containing 100 μM EGTA; (C) addition of 2 mM Ni^{2+} prior to nigericin and TRH; (D) addition of vehicle (Veh).

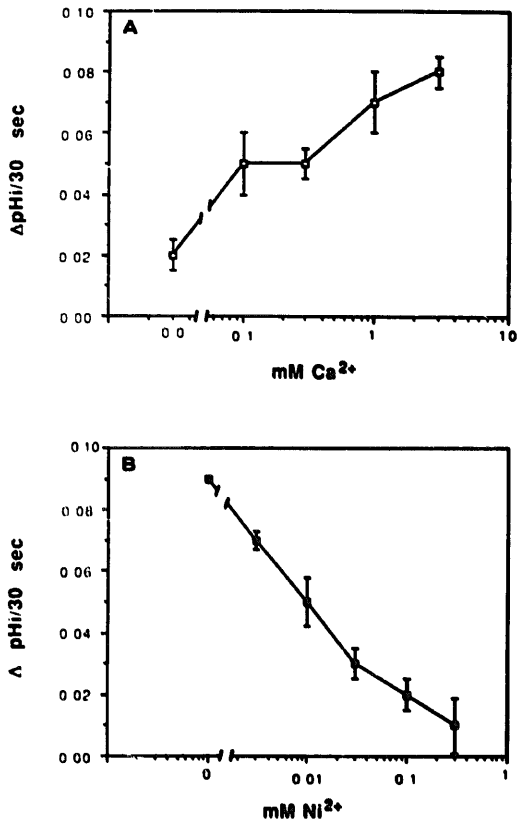


Fig. 6. Dose-dependent effect of extracellular Ca^{2+} and Ni^{2+} on TRH-induced activation of Na^+/H^+-exchange in GH_4C_1 cells. The cells were acidified in nominally Ca^{2+}-free buffer containing 100 μM EGTA as described in Fig. 5. (A) The appropriate concentration of extracellular Ca^{2+} was added to the cell suspension together with 100 nM TRH and the change in pH_i was measured. (B) The appropriate dose of Ni^{2+} was added prior to stimulating the cells with 100 nM TRH and the change in pH_i was measured. Each point is the mean ± S.E. of five determinations.

Discussion

In the present report, the basic properties of the TRH-induced activation of Na^+/H^+-exchange was characterized in GH_4C_1 cells. The basic characteristics of the Na^+/H^+-exchange were similar in GH_4C_1 cells to that observed in other cell systems [23,25,26]. Decreasing pH_o decreased Na^+/H^+-exchange in acid-loaded cells, suggesting that H^+-ions compete with Na^+ for binding to the antiport and that a H^+-gradient must exist in addition to a inward directed Na-gradient. Furthermore, no activation of Na^+/H^+-exchange was observed in response to TRH at basal pH_i-levels,

although a large Na^+-gradient was present in these experiments.

Stimulating Na^+/H^+-exchange in acid-loaded cells with TRH had a significant effect on the V_{max} value, compared with activation induced by Na^+ only. No difference was seen in the apparent K_m value. This could be the result of an increased affinity of the antiport for intracellular $[H^+]$ ($[H_i^+]$), as has been suggested to occur in several other cell systems in response to agonist-stimulation or osmotic shrinkage [23,25,27]. This is suggested to induce an alkaline shift in the pH_i-dependence of the antiport [25]. This suggestion is also supported by our recent study showing that in osmotically stressed GH_4C_1 cells TRH can activate Na^+/H^+-exchange at basal pH_i levels [13]. However, TRH had no effect on basal pH_i and a very low effect on Na^+/H^+-exchange when pH_i was close to basal levels, arguing against a shift in the pH_i-dependence. The results thus suggest, that in GH_4C_1 cells, an intracellular acidification is necessary for activation of Na^+/H^+-exchange. The increase in $[H_i^+]$ then probably affects the regulatory site on the antiport. TRH may enhance the binding of H^+ to either the transport site or the regulatory site on the antiport. Furthermore, the IC_{50} value for amiloride was approximately of the same magnitude when Na^+/H^+-exchange was activated with Na^+ only and with Na^+ together with TRH, suggesting that TRH did not affect the external binding site on the antiport. However, more detailed investigations on the regulatory effect of TRH on both intracellular and extracellular binding sites for Na^+ and H^+ on the antiport are obviously needed.

The present study also showed that extracellular Ca^{2+} had a significant effect on the TRH-induced activation of Na^+/H^+-exchange. We have recently reported that a transient increase in $[Ca^{2+}]_i$ can activate Na^+/H^+-exchange [2]. Furthermore, depletion of intracellular Ca^{2+} stores with ionomycin decreased the TRH-induced activation of exchange. The results in the present report suggest that, not only release of sequestered intracellular Ca^{2+}, but also influx of extracellular Ca^{2+} is of importance in the TRH-induced activation of Na^+/H^+-exchange. In several other cell types, like Swiss 3T3 cells [28] and WS-1 fibroblasts [29], a transient increase in $[Ca^{2+}]_i$ is sufficient to activate Na^+/H^+-exchange. In addition, influx of extracellular Ca^{2+} is needed for agonist-induced activation of Na^+/H^+-exchange in rat parotid acinar cells [30].

The mechanistic effect of Ca^{2+} on Na^+/H^+-exchange in GH_4C_1 cells is presently unknown. In a recent report by Kimura et al. [26], an increase in $[Ca^{2+}]_i$ was proposed to induce an alkaline shift in the cytosolic set point for activation of Na^+/H^+-exchange in platelets. The effect of Ca^{2+} may be due to activation of a Ca^{2+}-calmodulin pathway, as has been proposed to occur in smooth muscle cells [31], or to a direct effect of extracellular Ca^{2+} on the antiport. Our present study failed to observe an effect of extracellular Ca^{2+} per se on Na^+/H^+-exchange, in agreement with earlier studies [32,33]. However, considering the potent Na^+/Ca^{2+}-exchange in GH_4C_1 cells [34,35], an interaction between this system and Na^+/H^+-exchange can not be ruled out.

The TRH-induced activation of Na^+/H^+-exchange has been considered to be mediated via two mechanisms in GH_4C_1 cells: activation of PKC [1] and an increase in $[Ca^{2+}]_i$ [2]. Activation of Na^+/H^+-exchange via PKC is probably dependent on phosphorylation of the antiport [36] and in GH_4C_1 cells the PKC-induced activation seems to be independent of extracellular Ca^{2+} [2]. Stimulation of the cells with TRH increases $[Ca^{2+}]_i$ via two mechanisms: release of sequestered intracellular Ca^{2+} and influx of extracellular Ca^{2+} via activation of dihydropyridine-sensitive voltage operated Ca^{2+} channels, and a presently uncharacterized dihydropyridine-insensitive pathway [11, 15,37]. TRH-induced influx of extracellular Ca^{2+} via the dihydropyridine-insensitive pathway can, however, be inhibited with Ni^{2+} [38]. In the present report, inhibiting dihydropyridine-sensitive Ca^{2+} channels did not decrease TRH-induced activation of Na^+/H^+-exchange, whereas Ni^{2+} inhibited the TRH-induced activation of the antiport in a dose-dependent manner. However, the present experimental design cannot exclude the possibility that Ni^{2+} may have a direct inhibitory effect on the antiport molecule. The results thus seem to indicate that, in GH_4C_1 cells, influx of extracellular Ca^{2+} through a dihydropyridine-insensitive pathway is also necessary for the TRH-induced activation of Na^+/H^+-exchange in addition to release of sequestered Ca^{2+} and activation of PKC.

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References

1 Hallam, T.J. and Tashjian, A.H., Jr. (1987) Biochem. J. 242, 411–416.
2 Törnquist, K. and Tashjian, A.H., Jr. (1991) Endocrinology 128, 242–250.
3 Grinstein, S. and Rothstein, A. (1986) J. Membr. Biol. 90, 1–12.
4 Grinstein, S., Rotin, D. and Mason, M.J. (1989) Biochim. Biophys. Acta 988, 73–97.
5 Worley, P.F., Baraban, J.M., Supattapone, S., Wilson, V.S. and Snyder, S.H. (1987) J. Biol. Chem. 262, 12132–12136.

6 Guillemette, G. and Segui, J.A. (1988) Mol. Endocrinol. 2, 1249–1255.
7 Danthuluri, N.R., Donghee, K. and Brock, T.A. (1990) J. Biol. Chem. 265, 19071–19076.
8 Martin, T.F.J. (1983) J. Biol. Chem. 258, 14816–14822.
9 Macphee, C. and Drummond, A.H. (1984) Mol. Pharmacol. 25, 193–200.
10 Gershengorn, M.C. and Thaw, C. (1983) Endocrinology 113, 1522–1524.
11 Albert, P.R. and Tashjian, A.H., Jr. (1984) J. Biol. Chem. 259, 15350–15363.
12 Drust, D.S. and Martin, T.F.J. (1984) J. Biol. Chem. 259, 14520–14530.
13 Törnquist, K. and Stewen, P. (1990) Biochem. Biophys. Res. Commun. 172, 913–918.
14 Mariot, P., Sartor, P., Audin, J. and Dufy, B. (1991) Life Sci. 48, 245–252.
15 Törnquist, K. and Tashjian, A.J. (1992) Endocrinology 130, 717–725.
16 Tashjian, A.H., Jr., Yasumura, Y., Levine, L., Sato, G.H. and Parker, M.L. (1968) Endocrinology 82, 342–352.
17 Tashjian, A.H., Jr. (1979) Methods Enzymol. 58, 527–535.
18 Rink, T.J., Tsien, R.Y. and Pozzan, T. (1982) J. Cell. Biol. 95, 189–196.
19 Roos, A. and Boron, W.F. (1981) Phys. Rev. 61, 296–434.
20 Grinstein, S., Cohen, S. and Rothstein, A. (1984) J. Gen. Physiol. 83, 765–787.
21 Boron, W.F. (1977) Am. J. Physiol. 233, C61-C73.
22 Berke, B.C., Brock, T.A., Gimbrone, J., M.A. and Alexander, R.W. (1987) J. Biol. Chem. 262, 5065–5072.
23 Green, J., Yamaguchi, D.T., Kleeman, C.R. and Muallem, S. (1988) J. Gen. Physiol. 92, 239–261.
24 Green, J., Yamaguchi, D.T., Kleeman, C.R. and Muallem, S. (1988) J. Biol. Chem. 263, 5012–5015.
25 Grinstein, S., Cohen, S. and Rothstein, A. (1985) J. Gen. Physiol. 85, 765–787.
26 Kimura, M., Gardner, J.P. and Aviv, A. (1990) J. Biol. Chem. 265, 21068–21074.
27 Moolenaar, W.H., Tsien, R.Y., Van der Saag, P.T. and De Laat, S.W. (1983) Nature 304, 645–648.
28 Hesketh, T.R., Moore, J.P., Morris, J.D.H., Taylor, M.V., Rogers, J., Smith, G.A. and Metcalfe, J.C. (1985) Nature 313, 481–484.
29 Hendey, B., Mamrack, M.D. and Putnam, R.W. (1989) J. Biol. Chem. 264, 19540–19547.
30 Manganel, M. and Turner, R.J. (1990) J. Biol. Chem. 265, 4282–4289.
31 Little, P.J., Weissberg, P.L., Cragoe, E.J., Jr. and Bobik, A. (1988) J. Biol. Chem. 263, 16780–16786.
32 Moolenaar, W.H., Boonstra, J., Van der Saag, P.T. and De Laat, S.W. (1981) J. Biol. Chem. 256, 12883–12887.
33 Paris, S. and Pouyssegur, J. (1982) J. Biol. Chem. 258, 3503–3508.
34 Kaczorowski, G.J., Costello, L., Dethmers, J., Trumble, M.J. and Vandlen, R.L. (1984) J. Biol. Chem. 259, 9395–9403.
35 Törnquist, K. and Tashjian, A.H., Jr. (1989) Endocrinology 124, 2765–2766.
36 Sardet, C., Counillon, L., Franchi, A. and Pouyssegur, J. (1990) Science 247, 723–726.
37 Albert, P.R. and Tashjian, A.H., Jr. (1984) J. Biol. Chem. 259, 5827–5832.
38 Törnquist, K. (1991) Biochem. Biophys. Res. Commun. 180, 860–866.