Increased NHE1 expression is associated with serum deprivation-induced differentiation in immortalized rat proximal tubule cells

Luciene Regina Carraro-Lacroix,1 Marco Antonio Ramirez,2 Telma M. T. Zorn,3 Nancy Amaral Reboucas,4 and Gerhard Malnic1

Departments of 1Physiology and Biophysics and 3Cell Biology and Development, Institute of Biomedical Sciences, University of São Paulo, São Paulo, Brazil; and 2Biomedical Department, Faculty of Health Sciences, University of Antofagasta, Antofagasta, Chile

Submitted 15 July 2005; accepted in final form 10 February 2006

Carraro-Lacroix, Luciene Regina, Marco Antonio Ramirez, Telma M. T. Zorn, Nancy Amaral Reboucas, and Gerhard Malnic. Increased NHE1 expression is associated with serum deprivation-induced differentiation in immortalized rat proximal tubule cells. Am J Physiol Renal Physiol 291: F129–F139, 2006. First published February 21, 2006; doi:10.1152/ajprenal.00290.2005.—We studied the proton secretion mechanisms involved with pHi regulation in immortalized rat proximal tubule cells (IRPTC), a SV40-immortalized cell line derived from rat proximal tubule, and characterized the effects of serum deprivation on them. Using pHi, measurements with the fluorescent probe BCECF, we demonstrated that the IRPTC express both Na+/H+ exchanger and H+-ATPase, but only NHE1 is modulated by serum deprivation. In these cells, 24 h of serum starvation increased pHi from 7.08 ± 0.008 (n = 34) to 7.18 ± 0.018 (n = 33) as well as the pHi recovery rate from intracellular acidification with NH4Cl from 0.29 ± 0.022 pH U/min (n = 14) to 0.50 ± 0.024 pH U/min (n = 14), without modifying their buffering capacity. These effects were followed by several modifications in morphological features, indicating an increase in differentiation status. The altered activity of NHE1 was consistent with an increase of both transcription and translation of the antiporter, as the utilization of actinomycin D and cycloheximide significantly inhibited the upregulation of NHE1 induced by serum withdrawal. Inhibition of tyrosine phosphorylation by genistein blocked the serum deprivation-dependent activation of NHE. Moreover, the pharmacological inhibition of MEK1/2, the upstream activator of ERK1/2 by UO-126, significantly inhibited the stimulatory effect of serum starvation on Na+/H+ exchanger activity, whereas the putative p38 MAPK inhibitor SB-203580 failed to cause any effect on pHi recovery rates. Our findings indicate that during IRPTC differentiation by serum deprivation, there was a net enhancement of NHE1 activity. This upregulation of NHE1 by serum removal was consistent with an increase of RNA and protein synthesis of the exchanger, which depends on tyrosine kinase phosphorylation and ERK pathway activation.

Address for reprint requests and other correspondence: G. Malnic, Departamento de Fisiologia e Biofisica, Instituto de Ciencias Biomédicas, Universidade de São Paulo, Av. Prof. Lineu Prestes 1524, 05508-900 São Paulo, SP, Brazil (e-mail: gemalinic@usp.br).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
only the first mechanism is modulated by serum withdrawal. In these immortalized cells, morphological differentiation could be initiated by removal of serum from the culture media, accompanied by an enhancement in NHE1 activity. In addition, this serum deprivation-induced NHE1 upregulation is consistent, at least in part, with an increase of both transcription and translation of the exchanger, which depends on tyrosine kinase and MEK1/2 activation.

**MATERIALS AND METHODS**

**Materials**

IRPTC were provided by Dr. J. R. Ingelfinger (Pediatric Renal Research Laboratory, Massachusetts General Hospital, Boston, MA). DMEM, heat-inactivated feline bovine serum, sodium pyruvate, non-essential amino acids, and penicillin/streptomycin were purchased from Gibco (Grand Island, NY). Schering 28080 was obtained from Gerhard Giebisch (Yale University, New Haven, CT), BCECF-AM from Molecular Probes (Eugene, OR), actinomycin D from Calbiochem (San Diego, CA), N-ethylmaleimide from ICN Biomedicals (Irvine, CA), and S3226 and HOE-694 were provided by Jurgen Punter (Hoechst). Rabbit Na+/H+ exchanger-1 (NHE1) polyclonal antibody was purchased from Chemicon International (Temecula, CA). Anti-NHE1 (N1P1) was provided by J. Noel (Université de Montréal, Montreal, PQ). Anti-NHE3 (2B9) monoclonal antibody was kindly provided by D. Biemesderfer and P. Aronson (Yale University). The composition of the solutions used in this work is described in Table 1. The osmolality of solutions ranged from 290 to 310 mosmol/kg H2O.

**Measurement of pH, by fluorescence microscopy.** Intracellular pH was measured spectrofluorimetrically at 37°C with the fluorescent pH-sensitive probe, BCECF. Cells grown to confluence on glass coverslips were loaded with the dye by exposure for 5 min to 12 μM BCECF-AM in the control solution (solution 1). The acetoxymethyl ester form of BCECF enters the cell and is rapidly converted to the anionic-free acid form by intracellular esterases. After the loading period, the glass coverslips were rinsed with the control solution to remove the BCECF-containing solution and placed in a thermodrugged chamber mounted on an inverted epifluorescence microscope (Nikon, TMD). The measured area under the microscope had a diameter of 260 μm and contained on the order of 40 cells. Bathing solutions were rapidly exchanged without disturbing the position of the coverslips. Fluorescence was monitored using alternately 440 nm (pH insensitive) or 495 nm (pH sensitive) as excitation wavelengths utilizing a xenon light source. Emission was measured at 530 nm by a photomultiplier-based fluorescence system (Georgia Instruments, PMT-4000) at time intervals of 5 s, in pHs calculated from the fluorescence emission ratio of the two excitation wavelengths using a standard calibration procedure based on the use of 10 mM nigericin in high-potassium Ringer (solution 2; Table 1), at pH 5.91, 7.04, or 8.01 (39).

**Cell pH recovery.** Cell pH recovery was examined after the acidification of pHs with the NH4Cl pulse technique (6) after 2-min exposure to 20 mM NH4Cl (solution 3; Table 1), in the following situations: in the presence of external 145 mM Na+ (control; solution 1); or in the absence of external Na+ (solution 4; Table 1), with or without several inhibitors, as described later. In all the experiments, we calculated the initial rate of pH recovery (dPH/dt, pHi/min) from the first 2 min after the start of the pH recovery curve by linear regression analysis.

**Determination of intrinsic buffering capacity.** Intracellular buffer capacity (β) was determined using the technique described by Boyarsky et al. (7) and calculated as described by Weintraub and Machen (48). In our system, β refers to the ability of intracellular components excluding HCO3-/CO2 to buffer changes in pHi, and thus estimations of β were carried out using HEPES-buffered solutions. The H+ membrane transporters were blocked by a 0 mM Na+ solution plus 0.1 μM concanamycin (CCM), because β is most precisely estimated in cells whose pH+-regulatory mechanisms are blocked. After removal of Na+ from the external medium, the cells were exposed to a HEPES-buffered solution containing 50 mM NH4Cl, which was then stepwise reduced to 1 mM (in 50, 20, 10, 5, 2.5, and 1 mM steps). Calculation of β was performed according to the formula β = Δ[H+]i/ΔpHi, where the intracellular NH4+ concentration ([NH4+]i) was calculated from the Henderson-Hasselbalch equation on the assumption that NH4+ = NH3+.

**Analysis of Na+/H+ exchanger isoform expression by RT-PCR.** Total RNA was isolated from IRPTC by using the guanidine method (11) with some modifications. mRNA purification from total RNA was made with a Oligotex mRNA kit using the procedures described by the manufacturer (Qiagen). First-strand cDNA synthesis was carried out using the SuperScript Preamplification System as recommended by the manufacturer (Invitrogen). Primers for rat NHE1, NHE2, NHE3, and NHE4 were designed from published sequences (GenBank accession nos. NM_012652, NM_012653; NM_012654, and NM_173098, respectively; NHE1 sense, 5'-TCAGACCCACTG-GCCATATGAA-3', antisense, 5'-TCATTTGGTCCCGGGGGTCAA-3'; NHE2 sense, 5'-AAGCTTCCCGAGAAGTGTCAGA-3', antisense, 5'-GAGACGAGGCGTCTGCCCAA-3'; NHE3 sense, 5'-ATTCCTAATGGGAAAGTCTGCTAAC-3', antisense, 5'-AATGGATCTGACGCCGCCGAA-3'; NHE4 sense, 5'-TTGAGGAGACCTGGCGGAAA-3', antisense, 5'-TTGGCCCCCGATCCCGCAA-3'). For NHE8, we used degenerated oligonucleotides, designed for mouse NHE8 [GenBank accession no. AF482993; NHE8 sense, 5'-AA-GACTC(T/C/A)GAGAAGATGGGAA(A/G)/C(GG)/G(G/A)CGCCTG/G(C/A)CC33'].

<table>
<thead>
<tr>
<th>Table 1. Composition of solutions</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Solution 1</strong></td>
</tr>
<tr>
<td>NaCl 141</td>
</tr>
<tr>
<td>KCl 5.4</td>
</tr>
<tr>
<td>CaCl2 1</td>
</tr>
<tr>
<td>KH2PO4 0.3</td>
</tr>
<tr>
<td>MgCl2 0.5</td>
</tr>
<tr>
<td>MgSO4 0.5</td>
</tr>
<tr>
<td>NaHCO3 0.3</td>
</tr>
<tr>
<td>NaHPO4 0.3</td>
</tr>
<tr>
<td>HEPES 10</td>
</tr>
<tr>
<td>Glucose 0.6</td>
</tr>
<tr>
<td>NH4Cl 20</td>
</tr>
<tr>
<td>NMDG 141.3</td>
</tr>
<tr>
<td>pH 7.4</td>
</tr>
<tr>
<td><strong>Solution 2</strong></td>
</tr>
<tr>
<td>(K-HEPES)</td>
</tr>
<tr>
<td>NaCl 141</td>
</tr>
<tr>
<td>KCl 5.4</td>
</tr>
<tr>
<td>CaCl2 1</td>
</tr>
<tr>
<td>KH2PO4 0.3</td>
</tr>
<tr>
<td>MgCl2 0.5</td>
</tr>
<tr>
<td>MgSO4 0.5</td>
</tr>
<tr>
<td>NaHCO3 0.3</td>
</tr>
<tr>
<td>NaHPO4 0.3</td>
</tr>
<tr>
<td>HEPES 10</td>
</tr>
<tr>
<td>Glucose 0.6</td>
</tr>
<tr>
<td>NH4Cl 20</td>
</tr>
<tr>
<td>NMDG 141.3</td>
</tr>
<tr>
<td>pH 7.4</td>
</tr>
<tr>
<td><strong>Solution 3</strong></td>
</tr>
<tr>
<td>(NH4Cl)</td>
</tr>
<tr>
<td>NaCl 141</td>
</tr>
<tr>
<td>KCl 5.4</td>
</tr>
<tr>
<td>CaCl2 1</td>
</tr>
<tr>
<td>KH2PO4 0.3</td>
</tr>
<tr>
<td>MgCl2 0.5</td>
</tr>
<tr>
<td>MgSO4 0.5</td>
</tr>
<tr>
<td>NaHCO3 0.3</td>
</tr>
<tr>
<td>NaHPO4 0.3</td>
</tr>
<tr>
<td>HEPES 10</td>
</tr>
<tr>
<td>Glucose 0.6</td>
</tr>
<tr>
<td>NH4Cl 20</td>
</tr>
<tr>
<td>NMDG 141.3</td>
</tr>
<tr>
<td>pH 7.4</td>
</tr>
<tr>
<td><strong>Solution 4</strong></td>
</tr>
<tr>
<td>(0 Na+)</td>
</tr>
<tr>
<td>NaCl 141</td>
</tr>
<tr>
<td>KCl 5.4</td>
</tr>
<tr>
<td>CaCl2 1</td>
</tr>
<tr>
<td>KH2PO4 0.3</td>
</tr>
<tr>
<td>MgCl2 0.5</td>
</tr>
<tr>
<td>MgSO4 0.5</td>
</tr>
<tr>
<td>NaHCO3 0.3</td>
</tr>
<tr>
<td>NaHPO4 0.3</td>
</tr>
<tr>
<td>HEPES 10</td>
</tr>
<tr>
<td>Glucose 0.6</td>
</tr>
<tr>
<td>NH4Cl 20</td>
</tr>
<tr>
<td>NMDG 141.3</td>
</tr>
<tr>
<td>pH 7.4</td>
</tr>
</tbody>
</table>

All values are in mM, except for pH. NMDG, N-methyl-d-glucamine. HCl or NaOH was used in all Na+-containing solutions to titrate to the appropriate pH, and KOH was used in the Na+-free solution.
RESULTS

Characterization of the Proton Extrusion Systems Present in IRPTC and the Influence of Serum Deprivation

To study the H\(^+\) extrusion systems present in IRPTC and the influence of serum deprivation on them, we examined the intracellular pH recovery after acidification of pH\(_i\), with the NH\(_4\)Cl pulse technique. Figure 1A (A\(_1\) and A\(_2\)) shows two representative experiments in which IRPTC were first bathed with a 141 mM Na\(^+\) solution, exhibiting the basal pH\(_i\). After 2 min of exposure to NH\(_4\)Cl, during which the cell pH\(_i\) increased transiently, NH\(_4\)Cl removal caused a rapid acidification of pH\(_i\) as a result of NH\(_3\) efflux. In nondeprived cells, the pH\(_i\) recovery rate in the first 2 min was 0.29 ± 0.022 pH U/min (n = 14). Removal of serum from culture medium 24 h before the experiments resulted in a significant increase of the rate of pH\(_i\) recovery to 0.50 ± 0.024 pH U/min (n = 14). Serum starvation for periods of 6 to 48 h before the experiments caused the alterations in dPH/dt shown in Fig. 1B, indicating that the maximum increase occurred at 24 h deprivation. For this reason and to optimize the stimulatory response, in all experiments in which we studied the influence of serum deprivation on the proton extrusion systems of IRPTC, we performed serum deprivation for 24 h. Figure 1C shows the comparison between the first two situations, where the serum deprivation significantly stimulated the pH\(_i\) recovery in IRPTC.

Table 2 summarizes the main values of pH\(_i\) responses found in some of the studied experimental groups. Considering all the results from this work, IRPTC in pH 7.4 HCO\(_3\)-free solution have a mean baseline pH\(_i\) of 7.08 ± 0.008 (n = 34) in nondeprived cells and 7.18 ± 0.018 (n = 33) in 24-h serum-deprived cells (P < 0.0001). In serum-deprived cells, the steady-state pH\(_i\) in the Na\(^+\)-HEPES-buffered solution is higher than in nondeprived cells, which was observed in all experimental groups.

Figure 2 shows the effect of the absence of external Na\(^+\) and of the Na\(^+\)/H\(^+\) exchanger inhibitor, 5-N,N-hexamethylene) amiloride (HMA; 0.1 mM), on the pH\(_i\) recovery rates in nondeprived and serum-deprived cells. In the absence of Na\(^+\) or in the presence of HMA, the pH\(_i\) recovery rates were significantly reduced compared with controls in both situations (and pH\(_i\) recovery was not complete; Table 2), indicating that the pH\(_i\) recovery is mostly dependent on the Na\(^+\)/H\(^+\) exchanger. However, even in the absence of Na\(^+\), a significant rate of pH\(_i\) recovery was still observed in both conditions, indicating the presence of at least one Na\(^+\)-independent proton extrusion system in IRPTC. This behavior has been shown before in other PT cells, and evidence was presented suggesting that it was not due to the activity of the H\(^+\)-K\(^+\)ATPase, because in the groups where HMA plus Schering 28080 (a specific inhibitor of H\(^+\)-K\(^+\)-ATPase) were used, the pH\(_i\) recovery rates were similar to the HMA groups. The behavior observed in nondeprived and in serum-deprived cells was similar, showing that the cells express the Na\(^+\)/H\(^+\) exchanger as their main H\(^+\) extrusion system, but also a Na\(^+\)-independent mechanism. The pH\(_i\) recovery by this Na\(^+\)-independent system in serum-deprived cells was not significantly different from controls, indicating that the stimulatory effect of serum deprivation is on the Na\(^+\)/H\(^+\) exchanger.

Because several studies have shown the presence of H\(^+\)-ATPase in PT cells (2, 8), the next step was to evaluate the presence of this H\(^+\) extrusion system in IRPTC. Figure 3 shows the effects of N-ethylmaleimide (NEM; 0.5 mM) and concanamycin (0.1 μM), inhibitors of H\(^+\)-ATPase on Na\(^+\)-independent pH\(_i\) recovery from an acid pulse. In nondeprived cells, the utilization of these inhibitors reduced significantly the pH\(_i\) recovery to the same extent. The addition of EGTA to NEM did not change the recovery rate compared with the group where the sulfhydryl agent was used alone, indicating that extracellular calcium has no effect on the Na\(^+\)-independent mechanism. In serum-deprived cells, the pH\(_i\) recovery was reduced in the presence of concanamycin, confirming the presence of H\(^+\)-ATPase also in this experimental condition. In
all these groups where Na\(^+\)-free solutions were used to inhibit the Na\(^+\)/H\(^+\) exchanger, the pH\(_i\) after the acid load was lower than in control situations, because whereas the Na\(^+\)/H\(^+\) exchanger starts acting immediately after the change of the solutions, increasing the measured pH, H\(^+\)-ATPase is activated only approximately 2 min after solution change. For this reason, in Na\(^+\)-free conditions, we analyzed the rate of pH\(_i\) recovery in two moments: after 2 min of acid load, when H\(^+\)-ATPase starts acting, and at the same pH\(_i\) at which the dpH/dt was measured in control situations. In both cases, the pH\(_i\) recovery reached the same values, because the profile of pH\(_i\) recovery by H\(^+\)-ATPase is constant, following a straight line. Therefore, in the H\(^+\)-ATPase experiments, dpH/dt was measured 2 min after the start of recovery.

Table 2. Summary of pH\(_i\) responses in IRPTC to the addition of different solutions after an acute acid load

<table>
<thead>
<tr>
<th>Condition</th>
<th>Basal pH(_i)</th>
<th>pH(_i) After Acid Load</th>
<th>pH(_i) Recovery</th>
<th>dpH/dt</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nondeprived cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>7.10±0.013</td>
<td>6.41±0.044</td>
<td>7.20±0.067</td>
<td>0.29±0.0220</td>
<td>14</td>
</tr>
<tr>
<td>Na(^+) (0 mM)</td>
<td>7.08±0.012</td>
<td>5.96±0.058(^a)</td>
<td>6.80±0.035(^b)</td>
<td>0.07±0.0110</td>
<td>11</td>
</tr>
<tr>
<td>HMA (0.1 mM)</td>
<td>7.08±0.015</td>
<td>5.85±0.130(^a)</td>
<td>6.62±0.120(^a)</td>
<td>0.08±0.0140</td>
<td>9</td>
</tr>
<tr>
<td>HMA (0.1 mM) + Schering (10 (\mu)M)</td>
<td>7.09±0.020</td>
<td>5.65±0.069(^a)</td>
<td>6.51±0.110(^a)</td>
<td>0.08±0.0120</td>
<td>10</td>
</tr>
<tr>
<td>Na(^+) (0 mM) + NEM (0.5 mM)</td>
<td>7.04±0.012</td>
<td>5.69±0.043(^a)</td>
<td>5.93±0.140(^a)</td>
<td>0.02±0.0032</td>
<td>7</td>
</tr>
<tr>
<td>Na(^+) (0 mM) + NEM (0.5 mM) + EGTA (2.5 mM)</td>
<td>7.05±0.016</td>
<td>5.79±0.093(^a)</td>
<td>6.12±0.130(^d)</td>
<td>0.02±0.0035</td>
<td>6</td>
</tr>
<tr>
<td>Na(^+) (0 mM) + CCM (0.1 (\mu)M)</td>
<td>7.06±0.016</td>
<td>5.63±0.091(^a)</td>
<td>5.93±0.260(^a)</td>
<td>0.01±0.0049</td>
<td>6</td>
</tr>
<tr>
<td>Serum-deprived cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control, 24-h deprivation</td>
<td>7.18±0.037</td>
<td>6.34±0.029</td>
<td>7.32±0.063</td>
<td>0.50±0.0240</td>
<td>14</td>
</tr>
<tr>
<td>Control, 48-h deprivation</td>
<td>7.13±0.030</td>
<td>6.34±0.062</td>
<td>7.34±0.065</td>
<td>0.33±0.0280</td>
<td>6</td>
</tr>
<tr>
<td>Na(^+) (0 mM), 24-h deprivation</td>
<td>7.18±0.035</td>
<td>5.69±0.059(^a)</td>
<td>6.41±0.066(^c)</td>
<td>0.06±0.0060</td>
<td>11</td>
</tr>
<tr>
<td>HMA (0.1 mM), 24-h deprivation</td>
<td>7.18±0.039</td>
<td>5.60±0.048(^a)</td>
<td>5.95±0.140(^c)</td>
<td>0.06±0.0095</td>
<td>12</td>
</tr>
<tr>
<td>HMA (0.1 mM) + Schering (10 (\mu)M), 24-h deprivation</td>
<td>7.15±0.033</td>
<td>5.67±0.140(^a)</td>
<td>5.86±0.140(^d)</td>
<td>0.06±0.0130</td>
<td>8</td>
</tr>
<tr>
<td>Na(^+) (0 mM) + CCM (0.1 (\mu)M), 24-h deprivation</td>
<td>7.16±0.041</td>
<td>5.62±0.048(^c)</td>
<td>5.83±0.072(^c)</td>
<td>0.01±0.0037</td>
<td>5</td>
</tr>
</tbody>
</table>

Values are means ± SE; n is the number of observations; dpH/dt is the pH\(_i\) recovery rate in the first 2 min after acid load. \(^a\)P < 0.001 and \(^b\)P < 0.01 vs. control. \(^c\)P < 0.001 and \(^d\)P < 0.01 vs. Na\(^+\) (0 mM). \(^e\)P < 0.001 vs. control, 24-h deprivation. \(^f\)P < 0.001 vs. Na\(^+\) (0 mM), 24-h deprivation.
Isoforms of Na+/H+ Exchanger Modulated By Serum Deprivation Present in IRPTC

To analyze the Na+/H+ exchanger isoforms present in IRPTC that are modulated by serum, we used two inhibitors, HOE-694 (5 μM) for NHE1 and S3226 (1 μM) for NHE3 (12, 46), in nondeprived and serum-deprived cells. Figure 4 shows the pH_i recovery rates in serum-deprived cells. The utilization of S3226 did not affect the pH_i recovery compared with control, as the pH_i recovery rate in the first 2 min was 0.57 ± 0.035 pH U/min (n = 6), indicating the absence of NHE3 in IRPTC. However, with HOE-694, the pH_i recovery was significantly reduced to a value similar to those of Na+/H+ -free solutions plus 0.1 μM concanamycin (0.019 ± 0.0070 pH U/min, n = 10). This finding shows that the only Na+/H+ exchanger isoform present in IRPTC and that is modulated by serum deprivation is NHE1. The same behavior was observed also in nondeprived cells (data not shown).

To confirm these data, we performed RT-PCR in nondeprived and serum-deprived cells, using primers to several Na+/H+ exchanger isoforms commonly expressed in epithelial tissues (NHE1–4 and NHE8). Figure 5 shows the results obtained from serum-deprived cells, where PCR yielded products of the expected molecular weight only with the NHE1 primer (248 bp), confirming the presence of only this isoform in IRPTC. Similar results were obtained also in nondeprived cells (data not shown). Bands of the predicted sizes were detected for isoforms NHE1 (248 bp), NHE3 (329 bp), and NHE4 (213 bp) in cDNA samples from both the cortex and medulla of rats, illustrating the quality of the primers utilized.

To confirm the Na+/H+ exchange proteins expressed in IRPTC, cell lysates were immunoblotted for NHE1 and NHE3 (Fig. 5B). NHE1 was revealed with a polyclonal antibody, which recognized an ~85-kDa protein, whereas NHE3 was not detected, confirming the previous results. In rat kidney, anti-NHE3 labeled an ~80-kDa protein, showing the quality of the antibody.

Effect of Serum Deprivation on Bi

βi is an important mechanism to maintain pH_i at a constant level during acute pH_i displacements. In Fig. 6, calculated βi is plotted vs. pH_i at intervals of 0.2 U, each point representing the mean buffer capacity in these intervals. The solid line drawn through the points is the result of a fit to an exponential curve. βi depends significantly on pH_i over the tested pH_i range, increasing as pH_i is lowered. The data show that nondeprived and serum-deprived cells possess a similar βi, because we...
found no significant difference between corresponding points at the curves.

Influence of Serum Deprivation on the Morphology of IRPTC

Observations made on semiconfluent monolayers of IRPTC, which were cultured in the presence or absence of serum, suggested that morphological characteristics of the cells could be greatly influenced by medium composition. This was already visible at optical microscopy examination (data not shown). Electron microscopic studies of IRPTC grown in the presence of serum were poorly differentiated, as illustrated in Fig. 7, A and B. In general, the cells grown in medium with serum were characterized by lack of clear-cut polarization, rarity of microvilli, and of junctional complexes (Fig. 7 B). Our criteria of differentiation were the presence of apical microvilli, basally located nuclei, and evident junctional complexes between cells. Apical microvilli were always coexistent with basal nuclei, but junctional complexes were not always concomitant with the other two criteria. In a total of 22 nondeprived cells, 36.4% had such well-defined characteristics of differentiation. In contrast, cells grown in the absence of serum exhibited varying degrees of epithelial differentiation (Fig. 7 C, D, and E). IRPTC presented polarization properties, with the nucleus localized at the basolateral side of the cells, with microvilli covering the apical membrane, and with more developed tight junctions (Fig. 7 C). Out of 21 analyzed cells, 66.7% had such properties. Furthermore, serum-deprived cells showed more developed endoplasmic reticulum as well as Golgi apparatus (Fig. 7 D) and also a large amount and more developed nuclear pores (Fig. 7 E). However, some serum-deprived cells, as noted, displayed signs of degeneration, such as a large amount of vacuoles and lysosomes.

The role of NHE1 for cell differentiation was also evaluated by analyzing morphological changes when the cells were cultured in the presence of the NHE1 blocker HOE-694 (5 μM). When this experiment was performed, we observed, both with light and electron microscopy, that in 62.2% of serum-deprived cells (total of 37 cells) apical microvilli were apparent, whereas in similarly deprived cells but in the presence of
HOE-694, only 36.2% (n = 47) of cells had such properties. Similarly, basally located nuclei were found in 70.3% of serum-deprived cells, whereas only 29.8% of cells had basal nuclei in HOE-694-treated cells. When these differentiation criteria were analyzed statistically by the $\chi^2$-test, it was seen that differentiation was significantly ($P < 0.01$) associated with the treatments used, i.e., serum deprivation, no deprivation, and HOE-694. In addition, HOE-694-treated cells had large cytoplasmic vacuolization, extensive expansion of the perinuclear space, and widespread lack of intercellular junctions, impairing the formation of a continuous monolayer.

**Transcriptional and Translational Modulation of NHE1 by Serum Deprivation**

To determine whether RNA and protein synthesis are involved in the NHE1 stimulation by serum deprivation, IRPTC were treated with 0.1 $\mu$M actinomycin D (a transcription inhibitor) or 40 $\mu$M cycloheximide (a translation inhibitor) in the last 6 h of serum starvation, before the experiments. Unfortunately, we could not perform experiments in cells treated with actinomycin or cycloheximide for 24 h, which was the period of time used during starvation, because only a few cells could survive the treatment. Actinomycin D and cycloheximide did not cause any effect on $p$Hi recovery rates from acid load in nondeprived cells, but significantly inhibited the stimulatory effect of serum starvation on NHE1 activity (reaching $0.35 \pm 0.043$, $n = 6$ and $0.34 \pm 0.042$, $n = 6$ pH U/min, respectively, against a control value of $0.50 \pm 0.025$, $n = 5$ pH U/min, as shown in Fig. 8A). When these agents were added 30 min before the experiments, values of dpHi/dt were also reduced, but did not reach the control level (data not shown). Figure 8B shows that both NHE1 mRNA and protein levels are

![Fig. 7. Transmission electron microscopic analysis of serum-starved and nondeprived IRPTC. A and B: cells grown in the presence of serum displayed an apical surface devoid of well-defined microvilli and poor intercellular contacts. C-E: cells adapted to serum-free media formed a well-organized epithelium, with clear polarization: cells displayed an apical domain bearing microvilli and a basolateral domain showing tight junctions, well-developed Golgi apparatus, and nuclear pores (arrows). Magnifications: A, x5,000; B, x6,000; C, x4,000; D, x20,000; E, x15,000.](http://ajprenal.physiology.org/)

![Fig. 8. Transcriptional and translational modulation of NHE1 by serum deprivation. A: effects of actinomycin D (0.1 $\mu$M) and cycloheximide (40 $\mu$M) on initial rate of pH recovery following acute intracellular acidification in nondeprived and serum-deprived cells. *$P < 0.001$ vs. control, $\#P < 0.05$ vs. control of serum-deprived cells. B: effect of serum deprivation on NHE1 mRNA abundance ($B_1$) and protein expression ($B_2$). The levels of NHE1 mRNA were observed by RT-PCR in comparison to $\beta$-actin. NHE1 protein levels were detected by Western blotting, using specific antibodies for NHE1 (N1P1) in comparison to villin. ND and D, nondeprived and deprived cells, respectively.](http://ajprenal.physiology.org/)
increased after serum deprivation. β-Actin and villin were used as internal controls. Taken together, these results suggest a role of RNA and protein synthesis in regulating the net rate of pH\textsubscript{i} recovery mediated by NHE1 stimulated by serum deprivation.

**Involvement of Tyrosine Kinase and the MAPK Pathway in the Modulation of NHE1 by Serum Starvation**

To determine whether tyrosine kinase is involved with the stimulatory effect of serum deprivation on NHE1 activity, the tyrosine kinase inhibitor genistein (10 μM) was used. Cells were pretreated with the inhibitor for 30 min before the experiments. The data (see Fig. 9) show that in nondeprived cells, genistein has no intrinsic effects on pH\textsubscript{i} responses. Surprisingly, in serum-deprived cells, genistein completely blocked the stimulatory effect of serum starvation (reaching 0.32 ± 0.043 pH U/min, \( n = 7 \), compared with the control of 0.50 ± 0.026 pH U/min, \( n = 9 \)). These findings suggest that phosphotyrosine accumulation is required for the NHE1 stimulation induced by serum deprivation.

To investigate the potential functional significance of the activation of the ERK pathway by serum deprivation, we investigated the consequences of pharmacological inhibition of MEK1/2, the upstream activator of ERK1/2, using the inhibitor UO-126 (10 μM). Once again, exposure of nondeprived cells to the agent for 30 min before the experiments did not affect the pH\textsubscript{i} recovery rates, but significantly inhibited the stimulatory effect of serum starvation on Na\textsuperscript{+}/H\textsuperscript{+} exchanger activity (0.36 ± 0.047 pH U/min, \( n = 7 \)). These results show that the stimulation of NHE1 activity by serum deprivation is mediated by activation of the ERK pathway. To detect whether p38 MAPK was involved in this effect, we preincubated the cells with SB-203580 (10 μM), a potent inhibitor of p38 MAPK. We found that the p38 MAPK inhibitor failed to affect the pH recovery rates in both nondeprived or deprived cells (0.33 ± 0.028, \( n = 7 \) and 0.47 ± 0.025, \( n = 6 \) pH U/min, respectively), indicating that this protein is not involved in the stimulatory effect of NHE1 by serum deprivation. All these results are summarized in Fig. 9.

**DISCUSSION**

The purpose of this work was to determine the HCO\textsubscript{3}-independent proton extrusion systems present in IRPTC and their regulation by serum deprivation, a stimulus for cellular differentiation. The use of a HCO\textsubscript{3}-independent system permits to limit the H\textsuperscript{+} ion transporters to be studied, eliminating HCO\textsubscript{3} cotransporters and exchangers. Furthermore, the mechanisms underlying the different response in serum-deprived cells and the role of NHE in this process were analyzed. As most cell lines available are PT-like rather than PT per se and primary PT cells in culture have proven difficult to grow and passage, beyond dedifferentiating rapidly, a development of immortalized rat PT cell lines, such as IRPTC, is very useful. Therefore, IRPTC are a permanent cell line originated from the rat renal PT through transformation with temperature-sensitive A mutant virus of the simian virus 40 (tsA SV40) (38). According to Ingelfinger et al. (17), these cells polarize and express enzymes and proteins that are normally present in PT cells, thus displaying a highly differentiated phenotype. However, little is known about the regulation of cell pH in this cell line.

Our results concerning stationary cell pH and recovery from an ammonium pulse in nondeprived cells are comparable to data obtained with other proximal tubular cell lines, such as LLC-PK\textsubscript{1} (22). However, steady-state pH\textsubscript{i} in pH 7.4 HCO\textsubscript{3}-free solution increased with serum starvation, from 7.08 ± 0.008 (\( n = 34 \)) in nondeprived cells to 7.18 ± 0.018 (\( n = 33 \)) in 24-h serum-deprived cells. The pH\textsubscript{i} recovery rate in the first 2 min after the ammonium pulse was also higher in serum-starved cells, reaching 0.50 ± 0.024 pH U/min (\( n = 14 \)) after 24 h of serum deprivation, in contrast to 0.29 ± 0.022 pH U/min (\( n = 14 \)) in nondeprived cells. This indicates that serum deprivation stimulates mechanisms involved with pH regulation in IRPTC. It is not clear why the activation of NHE1 decreases after the peak at 24 h deprivation, but the cells, e.g., in the 48-h group, seem to suffer some degeneration and lose their ability to form a complete monolayer, suggesting that the prolonged incubation in serum-free medium might impair their viability.

The present study reports functional evidence for the presence of different proton secretion mechanisms, including the Na\textsuperscript{+}/H\textsuperscript{+} exchanger and H\textsuperscript{+}-ATPase in both nondeprived and serum-deprived IRPTC. The most important of these is the Na\textsuperscript{+}/H\textsuperscript{+} exchanger, because removal of extracellular Na\textsuperscript{+} or utilization of HMA (a Na\textsuperscript{+}/H\textsuperscript{+} exchanger inhibitor) led to an ~70% reduction in the rate of pH\textsubscript{i} recovery (Table 2) in nondeprived cells. These results are comparable with a number of studies by different authors (2, 3, 20, 30). As the present study was performed using impermeant glass supports, it was not possible to define the polarity of this proton transporter.

We were also able to show Na\textsuperscript{+}-independent pH\textsubscript{i} recovery after an acute acid load in our preparation (Fig. 3) in both nondeprived and deprived cells. Considering that the epithelium used in our study contains cells with characteristics of proximal cells, H\textsuperscript{+} ion-transporting ATPases were probably responsible for the Na\textsuperscript{+}-independent pH\textsubscript{i} recovery. In fact, with the use of a Na\textsuperscript{+}-free solution to which 0.5 mM NEM or

---

**Fig. 9.** Signaling pathways involved with the stimulatory effect of serum deprivation on NHE1 activity of IRPTC. Effects of genistein (10 μM), UO-126 (10 μM), and SB-203580 (10 μM). *P < 0.001 vs. control. #P < 0.01 and &P < 0.05 vs. control of serum-deprived cells.
0.1 μM concanamycin was added, the Na\(^+\)-independent pH recovery was almost completely inhibited, indicating the presence of a vacuolar H\(^+\)-ATPase. This observation agrees with the biochemical and functional results obtained by other authors (2, 24, 41). Moreover, in agreement with Wang et al. (47), we were unable to detect a component of Sch-28080-sensitive H\(^+\) extrusion, arguing against a significant role for H\(^+\)-K\(^+\)-ATPase in mediating PT acidification by IRPTC, although Sch-28080-sensitive K\(^+\)-ATPase activity has been detected in this nephron segment (49).

The Na\(^+\)-independent pH\(_i\) recovery was apparently lower in serum-deprived cells than in nondeprived cells. However, the difference among the groups was not significant, indicating that the proton extrusion system stimulated by serum withdrawal in IRPTC is only the Na\(^+\)/H\(^+\) exchanger. The isoform involved in this case is NHE1, because 1 μM S3226, a powerful inhibitor of NHE3 (35, 46), failed to inhibit the isoform in these cells, whereas 5 μM HOE-694 (a NHE1 inhibitor at this concentration) significantly inhibited the d\(\Delta\)H/d\(t\) by the same magnitude as did the 0 Na\(^+\) group. This was further substantiated by the presence of only NHE1 in RT-PCR and Western blot analysis.

Besides the ubiquitous NHE1, two additional isoforms were detected by other authors in PTs, NHE3 (the most abundant) and NHE8 (5, 14). NHE2 is also expressed in other proximal tubular cell lines, MCT and RKPC2 (9, 23), but not in plasma membrane fractions isolated from rat renal cortex (10). Studies in NHE3 null mice suggest that NHE3 is responsible for almost all HCO\(_3\)\(^-\) reabsorption mediated by Na\(^+\)/H\(^+\) exchange in the PT (47). However, the expression of NHE3, which is rapidly lost in primary culture as a consequence of the removal of the cells from their proper surrounding, appears to be also suppressed by immortalization (9). The present study suggests that IRPTC, an immortalized cell line, have also lost NHE3 expression, even in serum-deprived cells, where cells are expected to be more differentiated. Apparently, differentiated functions that may not be needed for survival in culture are likely to be lost in immortalized cells (9). Indeed, MCT and RKPC-2, other proximal-like tubule cell lines established from primary cultures using SV40, also do not express NHE3 (9, 23).

NHE1 was the first isoform to be cloned and plays a major role in intracellular pH homeostasis and cell volume regulation. An outstanding feature of NHE1 is that its activity can be modulated by a remarkably wide variety of stimuli, including growth factors, tumor promoters, hormones, and changes in cell volume or spreading (for reviews, see Refs. 25, 28, 43). A variety of signaling molecules is involved with NHE1 regulation by these stimuli, such as Ca\(^2+\)/calmodulin (42), low-molecular-weight GTPases Ras, and Rho (40), ERK1/2 kinases (4), calcineurin B homologous protein (29), and phosphatidylino- sitol 4,5 bisphosphate (1). A great deal of effort has been made to determine the interrelations among the functions of these signaling molecules leading to NHE1 activation, but the complete understanding of these processes remains unclear.

The Na\(^+\)/H\(^+\) exchanger is involved in cell proliferation and differentiation (15, 32). Our results, obtained from an immortalized cell line, suggest a possible involvement of serum withdrawal with increase of differential status of these cells. This was confirmed by ultrastructural analysis made by electron microscopy. Because IRPTC maintain their differentiation state without serum, it is possible that they may produce autocrine or paracrine factors that support their own survival. Among the immortalized cell lines that have been studied, several can be induced to differentiate by altering growth conditions or by adding trophic factors. In the catecholaminergic cell line Cath.a, differentiation could be initiated by serum deprivation (31). Moreover, P19 cells, an embryonal carcinoma cell line, can be induced to differentiate morphologically and biochemically by retinoic acid (45). In primary cultures of PT cells, cell differentiation is also modulated by medium composition (33). A number of studies have suggested a causal link between increased NHE1 activity and subsequent differentiation (13). In fact, the absence of Na\(^+\)/H\(^+\) exchanger as a pH-regulatory system can result in deficiencies in cell differentiation (45). We were able to show that cultivation of IRPTC in the presence of HOE-694, a specific blocker of NHE1 at the concentration of 5 μM, caused marked reduction of several differentiation criteria, such as the presence of apical microvilli, basally located nuclei, and formation of junctional complexes. Because differentiation may require either intracellular alkalization or increased Na\(^+\)/H\(^+\) exchange activity, it is possible that the mechanism to fulfill this requirement is increased Na\(^+\)/H\(^+\) exchanger gene expression followed by increased protein expression and activity (13).

Our findings suggest an involvement of RNA and protein synthesis in the NHE1 upregulation by serum deprivation in IRPTC, because in serum-deprived cells both NHE1 mRNA and protein were shown to be markedly increased. In addition, the inhibitors actinomycin and cycloheximide impaired the NHE1 stimulation of serum-deprived cells. Both these agents are antibiotics, actinomycin blocking RNA synthesis by complexing with DNA via deoxyguanosine residues, therefore inhibiting DNA-primed RNA polymerase, and cycloheximide blocking the translation of messenger RNA in cytosolic 80S ribosomes (26, 37). These results are in agreement with those from a number of other investigators (13, 32). These findings, together with an increase of the number of nuclear pores, as well as of their size, evidenced by ultrastructural analysis, suggest a more pronounced communication between nucleus and cytoplasm, agreeing with more protein synthesis. Nevertheless, in our system, we cannot exclude the involvement of other components, independent of NHE1 expression, in this modulation, such as regulatory proteins interacting with the cytoplasmic domain of NHE1 or direct phosphorylation of the antiporter.

In this study, we also examined the putative role of MAPKs on NHE1 modulation by serum deprivation. MAP kinases constitute a large family of Ser/Thr protein kinases activated by separate cascades, which in turn regulate multiple processes stimulated by extracellular agents. In mammalian cells, the first and best-characterized MAPK cascade is the ERK1/2. It involves activation of ERK1/2 by direct phosphorylation by MEK1 and MEK2, which are themselves phosphorylated and activated by the serine/threonine kinase Raf. Raf is recruited to the membrane by Ras on activation of either G protein-coupled receptors or tyrosine kinase receptors (4, 36). In our case, genistein completely blocked the stimulatory effect of serum deprivation on NHE1 activity, indicating the involvement of tyrosine kinase phosphorylation in this regulation. Moreover, U0-126 also inhibited the stimulation of NHE1 by serum deprivation, showing that MEK1/2, the upstream activator of ERK1/2, is related to the observed effect. Bianchini et al. (4,
In conclusion, our results suggest that serum deprivation results in an upregulation of the NHE present in IRPTC, NHE1, together with an increase in their differentiation status. This positive regulatory pathway depends on the activation of tyrosine kinases, on the ERK1/2 cascade and finally regulation of gene expression.

ACKNOWLEDGMENTS

The authors thank Dr. M. Oliveira-Souza, C. N. Alves Bezerra, and F. A. Correia Barrence for helpful support.

GRANTS

This work was supported by grants and fellowships from Fundação de Amparo a Pesquisa do Estado de São Paulo (99/128663) and CNPq.

REFERENCES