Human cytomegalovirus infection enhances osmotic stimulation of Na\(^+\)/H\(^+\) exchange in human fibroblasts

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Human cytomegalovirus infection enhances osmotic stimulation of Na\(^+\)/H\(^+\) exchange in human fibroblasts. Am. J. Physiol. 273 (Cell Physiol. 42): C1739–C1748, 1997.—Infection with human cytomegalovirus (HCMV) causes an enlargement (cytomegaly) of human fibroblasts (MRC-5). As a first step toward determining whether solute uptake, mediated in part by Na\(^+\)/H\(^+\) exchange, is responsible for the development of cytomegaly, we studied the effects of HCMV infection on intracellular pH (pHi) regulation (nominal CO\(_2\)/HCO\(_3\) concn = 0) by comparing cytomegalic cells with mock-infected cells. Seventy-two hours after HCMV infection of MRC-5 cells, we observed the following changes relative to mock-infected cells. Seventy-two hours after HCMV infection, the cell volume increase is osmotically driven; that is, the cell volume increase starts with the proposition that the osmotic content of osmotically active particles (osmolytes), either organic or inorganic solutes. It is possible that HCMV may increase host cell water space by either usurping host cell programs used for cellular enlargement in preparation for cell division (i.e., a mitogenic-like effect) or by disrupting steady-state cell volume maintenance.

Several observations support the view that an association exists between cytomegaly and alterations of ion transport mechanisms, in particular, transport mechanisms involving sodium (1). Evidence for enhanced Na\(^-\)/K\(^+\) pump activity after HCMV infection includes 1) a significantly increased ouabain-sensitive \(^{86}\)Rb\(^+\) uptake (31) and 2) a threefold increase in the number of ouabain-binding sites (Na\(^+\)-K\(^+\) pump sites) after HCMV infection (3). In addition, amiloride, an inhibitor of Na\(^+\) movements via Na\(^+\)/H\(^+\) exchange, also inhibits the development of cytomegaly (31). A role for conductive sodium entry cannot be completely ruled out in cytomegaly, although voltage-sensitive sodium currents disappear within 18 h after HCMV infection (4). Further circumstantial evidence linking sodium-dependent transporters to HCMV effects comes from the observation that the increase in the number of ouabain-binding sites after HCMV infection can be substantially reduced by incubating cells in media with reduced Na\(^+\) concentration ([Na\(^+\)]), or Cl\(^-\) concentration ([Cl\(^-\)]) or by treatment with amiloride or ethylisopropyl amiloride (Ref. 3). Finally, there is the observation that reduction of [Na\(^+\)] in the culture medium after HCMV infection inhibits the development of cytomegaly (31).

Preliminary findings have been presented in abstract form (2, 13).

METHODS

Cell cultures and HCMV infection. Human embryo lung fibroblasts (MRC-5, passages 18–30, were cultured in Eagle’s minimum essential medium with Earle’s salts, supplemented with 2 mM glutamine and 10% heat-inactivated fetal calf serum. The osmolality of the cell culture medium was adjusted to 285 mosmol/kgH\(_2\)O to match the osmolality of the experimental solutions (see Solutions and reagents). The cells were grown in an incubator with a humidified atmosphere of 5% CO\(_2\) in air at 37°C. A stock of HCMV [strain AD169, originally a generous gift from Dr. Thomas Albrecht (Department of Microbiology, University of Texas Medical Branch, Galveston, TX)] was generated in confluent MRC-5 cells (see Ref. 3 for more details).

Three days after seeding on 6 × 24-mm glass coverslips, confluent MRC-5 cells were exposed for 1 h to a suspension containing either HCMV at a multiplicity of infection of approximately three plaque-forming units per cell or a mock-infecting, virus-free suspension (see Ref. 3 for details of mock infection). Two days postexposure (PE) to HCMV, the fetal
calf serum was reduced to 1%, because reducing the fetal calf serum helped minimize fluorescent dye loss during experiments.

Solutions and reagents. Standard N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES)-buffered solution contained (in mM) 128 NaCl, 5 KCl, 1 MgCl2, 1 CaCl2, 10 glucose, and 20 HEPES. The solution pH was adjusted to 7.4 (at 37°C) with Na-methyl-d-glucamine (NMDG+), and the osmolality was 285 ± 5 mosmol/kg H2O. Sodium-free solution was the same as the standard HEPES-buffered solution except that NaCl was replaced with NMDG-Cl. Solutions with different Na+ concentrations were obtained by appropriately mixing the standard HEPES-buffered and the Na+-free solutions. The intracellular pH (pHi) calibration solution contained (mM) 130 potassium gluconate, 20 NMDG-Cl, 2 MgCl2, 20 HEPES, and 10 µM nigericin (Sigma, St. Louis, MO). When required, solutions were made 120 or 204% of normal osmolality by the addition of 57 or 297 mM sucrose, respectively. Diethyl amiloride (DEA; Molecular Probes, Corvallis, OR), a 5-amino-substituted derivative of amiloride (26), was prepared as a 10 mM stock in distilled water and used at a final concentration of 5 mM. This concentration was sufficient to block virtually all Na+/H+ exchanger (NHE) activity and avoided a paradoxical, unexplained alkalinization, which is sometimes observed when higher concentrations of amiloride derivatives are used (unpublished observations; Ref. 24).

Proton fluxes were calculated by multiplying dpHi/dt by the buffering power, by an index of cell volume, and by Avogadro’s constant (Nₐ). The difference in rates of acid loading was determined using the technique similar to that of Boyarsky et al. (7). Cells were simultaneously acid loaded and Na+ depleted by removing external Na+ (replaced with NMDG+). When the external Na+ was returned, the rate of change of pHi (dpHi/dt) was determined during the recovery from the acid load. The Savitzky-Golay procedure, built into TableCurve (Jandel, San Rafael, CA), was used to compute the smoothed derivative from the time trace.

The dpHi/dt for DEA-sensitive acid extrusion, presumably mediated by the NHE, was determined as the difference between the net rate of acid recovery and the rate of acid loading at corresponding pHi values. The rate of acid loading was computed using the period of DEA-induced acidification that followed the pHi recovery.

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External sodium dependence of the NHE. External sodium dependence was determined from the DEA-sensitive acid extrusion flux \( I_{\mathrm{NHE}} \) obtained at different extracellular \( [\mathrm{Na}^+] \) values during recovery from an acid load. In all cases, we determined the flux when \( \mathrm{pHi} = 7.3 \).

All data are reported as means ± SE. P values were calculated using an unpaired t-test.

RESULTS

Resting \( \mathrm{pHi} \) of HCMV- and mock-infected MRC-5 cells: evidence for \( \mathrm{Na}^+/\mathrm{H}^+ \) exchange and acid loading. We measured resting \( \mathrm{pHi} \) of both HCMV-infected and mock-infected MRC-5 fibroblasts 72 h PE. The external bathing solution was nominally free of \( \mathrm{HCO}_3^- \) and \( \mathrm{CO}_2 \). Under this condition resting \( \mathrm{pHi} \) of the mock-infected cells was 7.40 ± 0.02 (n = 114), a value similar to that reported for confluent MRC-5 fibroblasts by other workers (36). In contrast, the resting \( \mathrm{pHi} \) of HCMV-infected cells was 7.49 ± 0.01 (n = 100), a value significantly more alkaline than in mock-infected cells (P < 0.01). The preceding data were obtained using the high-[\( \mathrm{K}^+ \)]/nigericin technique of calibration. In a separate study, using null point calibrations, HCMV-infected cells were 0.23 pH units more alkaline than mock-infected cells (P < 0.001), and the resting \( \mathrm{pHi} \) values for both treatments were ~3% lower (more acidic) than those obtained using the high-[\( \mathrm{K}^+ \)]/nigericin technique. Therefore, it is clear that, regardless of the absolute magnitude of our reported \( \mathrm{pHi} \) values, a highly significant \( \mathrm{pHi} \) difference exists between HCMV-infected and mock-infected cells.

Resting \( \mathrm{pHi} \) is a dynamic property resulting from the algebraic sum of two competing processes: acid extrusion and acid loading (6). In the absence of extracellular \( \mathrm{HCO}_3^- \) acid extrusion is accomplished by the NHE in most mammalian cells, including fibroblasts (18). Therefore, one possible explanation for the more alkaline \( \mathrm{pHi} \) we report for HCMV-infected cells could be that the NHE is much more active in the HCMV-infected cells than in the mock-infected cells. If this were the case, blocking the NHE would cause the \( \mathrm{pHi} \) of the HCMV-infected cells to approach that of the mock-infected cells. We tested this possibility by comparing the effects on the \( \mathrm{pHi} \) of treating both HCMV-infected and mock-infected cells with DEA, a specific blocker of the NHE. As seen in Fig. 1, although treatment with 5 \( \mu \mathrm{M} \) DEA caused acidification in both HCMV-infected and mock-infected cells, the difference in \( \mathrm{pHi} \) between the two treatments was not abolished by exposure to DEA. Ten minutes after removal of DEA from the bathing solution, \( \mathrm{pHi} \) had recovered in both HCMV-infected and mock-infected cells (not shown). These results show that the NHE is functionally active in both cell types, and both infected and uninfected cells have functional acid-loading processes. However, the fact that the \( \mathrm{pHi} \) of the two cell types do not become equal after inhibition of the NHE strongly suggests that the difference in resting \( \mathrm{pHi} \) values for the two cell treatments is not simply the result of a more active NHE in the HCMV-infected cells and therefore suggests that a virally mediated effect on acid-loading processes might also be involved.

Effects of HCMV infection on \( \beta \). To quantitatively characterize properties of both the NHE and the acid-loading mechanisms we needed to determine their equivalent \( \mathrm{H}^+ \) fluxes. This determination requires knowledge of the \( \beta \) and its \( \mathrm{pHi} \) dependence. We measured these as described in METHODS. Figure 2 is a plot of the \( \mathrm{pHi} \) dependence of \( \beta \) in both HCMV-infected and mock-infected cells. Following the approach of Wilding et al. (41), the data from the mock-infected and HCMV-infected cells were analyzed by linear regression to determine the slope (\( \beta \)), which is the slope of the line through the data points. The slope is given by the expression \( \beta = k [\mathrm{H}^+]/(k + [\mathrm{H}^+]^n) \), where K is the dissociation constant of the intracellular buffer, and \( C \) is the concentration of the intracellular buffer. Symbols are means ± SE of binned data. In mock-infected cells (C), the fitting parameters were as follows: \( K = 10^{-6.88}, C = 48 \) mM, F value = 24. When the same data were fitted with a linear model, the following parameters were obtained: intercept = 142, slope = -18, F value = 17. In HCMV-infected cells (H), \( \beta \) was obtained over a more limited range of \( \mathrm{pHi} \). The data could be fitted equally well to either an equivalent intracellular buffer \( (K = 10^{-6.14}, C = 67 \) mM, F value = 22) or to a straight line (intercept = 141, slope = -18, F value = 21).
infected cells in Fig. 2 were fitted to a model with one single "equivalent" intracellular buffer. The fitted data reveal an acidic shift of the apparent acidic dissociation constant of the cellular buffer from 6.68 for the mock-infected cells to 6.14 for the HCMV-infected cells. Furthermore, Fig. 2 clearly shows that over the pHi range of the present study (7.1–7.5), HCMV-infected cells have about one-half of the β of the mock-infected cells. Similar decreases in β have been observed after SV40 transformation of MRC-5 (35).

Effects of HCMV infection on the pHi dependence of acid loading and acid extrusion. The more alkaline resting pHi of HCMV-infected cells under nominally acid loading and acid extrusion. The more alkaline the same preparation by using the following general cession and thereby uncover background acid-loading processes, suggesting that changes in acid-loading processes may also result from HCMV infection. Therefore we examined the effects of HCMV infection on these processes over a range of pHi values because the rates of both acid extrusion and acid loading are inherently pHi sensitive (6).

We measured both acid uptake and acid extrusion in the same preparation by using the following general protocol. First, we exposed the cells to Na1-free external solution to acid load and sodium deplete them. Once a pHi had reached a new steady state, extracellular Na1 was returned to activate the NHE, causing pHi to increase. Finally, DEA was applied to block acid extrusion and thereby uncover background acid-loading processes (see Fig. 1).

As seen in Fig. 3, removal of extracellular Na1 (NMDG1-replaced Na1+) caused the pHi of both the mock-infected and the HCMV-infected cells to acidify, but mock-infected cells acidified much faster and to a greater extent than HCMV-infected cells. In fact, HCMV-infected cells never acidified below pHi ~7.2 even after 30 min of Na1-free treatment.

In principle there are two general ways acid loading may occur: 1) metabolic production of proton equivalents and/or 2) unidentified proton-equivalent ion transport mechanisms, including "reverse" operation of the NHE (e.g., net H+ entry in exchange for cytoplasmic Na+). The latter possibility was ruled out because we observed no difference in the rates of acidification in the presence or absence of DEA. Regardless of the mechanism(s) of acid loading, it is clear that the acid-loading rate was reduced by HCMV infection. In fact, because the infection also reduces the β, the reduction of the acid-loading flux is even greater than the reduction of the dpHi/dt.

When external Na1 was returned, net acid extrusion occurred, causing pHi to recover in both cell types (Fig. 4). The background acid-loading process(es) was then revealed by treatment with 5 µM DEA. This way of measuring acid loading excludes any potential contribution of reverse NHE (see above). Thus, when external Na1 was returned, the increase in pHi (Fig. 4, segment a-b) was the algebraic sum of two processes: net acid extrusion (presumably via NHE) and net acid loading (Fig. 4, segment b-c). The mechanism(s) unknown).

Net proton-equivalent fluxes for both acid extrusion and acid loading were calculated from a series of experiments similar to those shown in Fig. 4. The pHi dependence of net acid extrusion and net acid-loading fluxes in the two cell types are shown in Fig. 5. It can be seen that HCMV infection resulted in an alkaline shift.
plays a central role in what is believed to be normal cell volume regulation by participating (together with the Cl⁻/HCO₃⁻ exchanger) in regulatory volume increases in response to cell shrinkage (e.g., Ref. 19). In view of the significantly larger volume of HCMV-infected cells, we tested whether HCMV infection altered the volume-sensitive properties of the NHE in MRC-5 cells. Cells were exposed to either a moderate (120% of normal osmolality, 345 mosmol/kgH₂O) or severe (204% of normal osmolality, 580 mosmol/kgH₂O; isosmotic = 285 mosmol/kgH₂O) hyperosmotic challenge induced by the addition of sucrose to the bathing medium. Experiments were performed in the absence or presence of DEA. Figure 7 illustrates the time course of pH₁ changes for both mock-infected and HCMV-infected cells in response to the hyperosmotic challenges. dpH₁/dt from the data presented in Fig. 7 were converted to equivalent H⁺ fluxes, and the collated results are plotted in Fig. 8.

Both the mock-infected and the HCMV-infected cells respond to a hyperosmotic challenge in a biphasic manner. First, both cell types exhibit a slight acidification (Fig. 7). Such initial, transient acidification during hyperosmotic challenge has also been observed by other investigators (25, 34). The cause is unknown, but may be due to either osmotically enhanced metabolic acid production (34) and/or changes in the pK₁ of intracellular buffers (15). In our cells, the maximum rate of this acidification occurred ~1 min after initiating the challenge and increased with the magnitude of the chal-

![Graph](image-url)

Fig. 5. pH₁ dependence of Na⁺/H⁺ exchange and acid loading. pH₁ dependence of the Na⁺/H⁺ exchanger (NHE) fluxes and "basal" acid-loading fluxes (DEA-insensitive acid loading) was obtained from experiments such as those illustrated in Fig. 4. Equivalent fluxes were calculated from measured rate of change of pH₁ (dpH₁/dt) as described in METHODS. J₁, net DEA-sensitive proton equivalent efflux; J₁, net proton equivalent influx.

in the pH₁ dependence for net DEA-sensitive acid extrusion (J₁) as well as a reduction in the rate of net acid loading (J₁). Thus the pH₁ at which the rate of net acid extrusion is equal to the rate of net acid loading is shifted in the alkaline direction by ~0.15 pH units in HCMV-infected cells compared with the mock-infected cells. These alkaline shifts in the pH₁ dependence for both net acid extrusion and net acid loading probably explain the more alkaline resting pH₁ of the HCMV-infected cells relative to the mock-infected cells.

Effect of HCMV infection on the external Na⁺ dependence of Na⁺/H⁺ exchange. We next examined the external Na⁺ dependence of the NHE in both mock- and HCMV-infected cells to further characterize the effects of HCMV infection on functional properties of the NHE. The external Na⁺ dependence of NHE flux (J₁) was obtained by varying extracellular [Na⁺] between 8 and 128 mM Na⁺ and calculating the flux at a pH₁ of 7.3. This pH₁ was selected because it is the most acidic pH₁ that can be reliably achieved for both cell treatments. Figure 6 shows the effects of infection with HCMV on the activation of DEA-sensitive H⁺ extrusion as a function of external [Na⁺]. When these data are fitted to the Michaelis-Menten equation, we see that the apparent maximal velocity (Vₘₐₓ) was increased by HCMV infection (HCMV infected = 165 ± 19.0; mock infected = 90.5 ± 9.0 H⁺·s⁻¹·ouabain-binding site⁻¹), whereas the apparent affinity for external Na⁺ [Kₐ₅(Na)₀] was reduced by the virus [HCMV infected Kₐ₅(Na)₀ = 79 ± 17 mM; mock infected Kₐ₅(Na)₀ = 25.3 ± 8.4 mM].

Effect of HCMV infection on the response to hyperosmotic challenge by the NHE. In many cells, the NHE
Interestingly, DEA largely blocked the acidification (Fig. 7).

After the initial acidification, there was a distinct difference between the hyperosmotic response of mock-infected and HCMV-infected cells as seen in Fig. 7. Mock-infected cells responded with a slight alkalinization. In fact, the alkalinization response of mock-infected cells to a moderate hyperosmotic challenge was barely detectable (Figs. 7A and 8). However, the response of the mock-infected cells to exposure to a severe hyperosmotic solution was significantly larger (Figs. 7B and 8). Under this latter condition, the rate of alkalinization (dpH/dt = 0.026 ± 0.005 pH units/min, n = 5) peaked ~2.5 min after the challenge. Offsetting effects of osmotically induced increases of both acid loading and NHE-mediated acid extrusion processes are unlikely to explain the low rate of intracellular alkalization for mock-infected cells because hyperosmotic treatment in the presence of DEA revealed little acidification.

In sharp contrast to the behavior of mock-infected cells, HCMV-infected cells responded to the increased osmolality with a significant alkalinization, whose rate increased with increased osmolality (see Figs. 7 and 8). Thus increasing the intensity of the hyperosmotic challenge to the HCMV-infected cells from 120% (n = 9) to 204% (n = 4) of normal osmolality resulted in a corresponding increase in the magnitude of the alkalinization change (~0.2–0.3 pH units; Fig. 7), an increase in the maximum rate of alkalinization (0.061 ± 0.008 to 0.212 ± 0.009 pH units/min), and a reduction in time for this value to be reached (3.5–2 min; Fig. 7). Although most of this alkalinization was via the NHE, there was a distinct DEA-insensitive alkalinization (~0.13 pH units; Figs. 7D and 8) in HCMV-infected cells after a severe hyperosmotic challenge. Because this response also occurred in the absence of bath Na^+ (replaced with NMDG^+), it is unlikely to be due to the activity of the NHE (data not shown). Regardless of the severity of the hyperosmotic challenge, when HCMV-infected cells were returned to an isosmotic bathing solution, pH_i slowly returned toward its initial, unstimulated value.

**Fig. 7.** Hyperosmotic activation of the NHE. Mock-infected (A and B) and HCMV-infected (C and D) cells were exposed for 10–15 min to a hyperosmotic challenge, either moderate (120% of normal osmolality, 345 mosmol/kgH_2O, 57 mM sucrose) or severe (204% of normal osmolality, 580 mosmol/kgH_2O, 297 mM sucrose) hyperosmotic challenge in the absence (closed circles) or presence (open circles) of DEA (5 µM). Error bars indicate SE of mean of individual measurements. Number of each kind of experiment is indicated in parentheses.

**Fig. 8.** Net osmotic stimulation of H^+ fluxes. Values for dpH/dt at 2 min after hyperosmotic challenge were converted to net osmotically stimulated H^+ fluxes (J_{hyperosmotic} - J_{isosmotic}; see METHODS for calculation of fluxes; positive values denote a net H^+ efflux). In comparison with the mock-infected cells, the HCMV-infected cells exhibit a much larger osmotically stimulated H^+ flux. This osmotically stimulated efflux was DEA sensitive, and its magnitude was dependent on the degree of the hyperosmotic challenge.
The higher dpH/dt we report for HCMV-infected cells relative to mock-infected cells might simply be a consequence of the lower $\beta$ (Fig. 3) of the HCMV-infected cells. However, the proton flux is calculated using $\beta$ (as well as cell surface area and volume; see METHODS) and hence reflects true transmembrane H$^+$ movements. Although we have not measured $\beta$ under hyperosmotic conditions, other investigators have done so and found that it increases (e.g., Ref. 15) in proportion to the decrease of cell volume such that the product of $\beta \times$ cell volume remains constant (29). On the assumption that the same relationship holds for the cells we studied, we calculated the net H$^+$ fluxes from the data presented in Fig. 7 and plotted the results in Fig. 8. Although the net H$^+$ fluxes in mock-infected cells were stimulated in an osmolarity-dependent manner by hyperosmotic challenge, the same osmotic challenge resulted in fluxes that were 5–10 times greater in the HCMV-infected cells. In addition, the relative change in net flux when the osmotic stimulus was increased from 120 to 204% was greater in the HCMV-infected cells. Because DEA blocked much of the stimulation of net H$^+$ flux, it is clear that HCMV infection dramatically increased the osmosensitivity of the net H$^+$ flux mediated by the NHE.

**DISCUSSION**

HCMV infection causes an alkaline shift in the pH$_i$ dependence of the NHE. Seventy-two hours after exposure to HCMV, MRC-5 cells have a steady-state pH$_i$ that is $\sim 0.1$–0.2 pH unit more alkaline than that measured in mock-infected MRC-5 cells in the nominal absence of CO$_2$/HCO$_3$$. Steady-state resting pH$_i$ is determined by the algebraic sum of two categories of processes: acid extrusion and acid loading. In the present work we identified virally induced changes to both these categories of processes. We show that the pH$_i$ dependence of the NHE is shifted in the alkaline direction by $\sim 0.1$ pH unit (Fig. 4), whereas the mechanism(s) responsible for acid loading is strongly reduced in the HCMV-infected cells (Fig. 4). Thus the apparent alkaline shift of the pH$_i$ dependence of the NHE, coupled with the reduction of the acid-loading fluxes (Fig. 3), probably accounts for the more alkaline resting pH$_i$ we observed under nominally CO$_2$/HCO$_3$$-$free conditions. Others have reported virally mediated effects on pH$_i$ (20, 22). Of particular interest to the present work is a report that the human immunodeficiency virus envelope glycoprotein gp120 stimulates Na$^+$/H$^+$ exchange (5). Further work will be required to determine if effects on pH$_i$ and its regulation is a common viral infection theme.

Table 1 summarizes the effects of HCMV infection on several key properties related to pH$_i$ regulation. With the use of previous estimates of volume and surface area (3), the measured $\beta$, and the measured dpH/dt values, we calculated the flux through the NHE and through the acid-loading pathway(s) at pH$_i$ = 7.45. This pH$_i$ value was chosen because it is the resting pH$_i$ for both mock-infected and HCMV-infected cells in the presence of CO$_2$/HCO$_3$ (14), a more “physiological” condition.

The information in Table 1 gives us two important insights into the effects of HCMV infection. First, infection increases NHE activity while decreasing activity of the non-CO$_2$/HCO$_3$-dependent acid-loading process(es). Second, as we have shown, cells bathed in CO$_2$/HCO$_3$-free media have significantly different resting pH$_i$ values, whereas both mock-infected and HCMV-infected cells bathed in CO$_2$/HCO$_3$-containing media have the same resting pH$_i$ of $\sim 7.45$ (14). This means that, in the presence of CO$_2$/HCO$_3$$_{-}$, both cells achieve a steady state with regard to acid extrusion and acid-loading processes at the same pH$_i$. For this to be true, there must be a CO$_2$/HCO$_3$$_{-}$-dependent acid-loading process that is also stimulated by HCMV infection. We have preliminary evidence that this CO$_2$/HCO$_3$$_{-}$-dependent process is Cl$^-$/HCO$_3$$_{-}$ exchange (14).

Acid loading and HCMV infection. It is only recently that the possibility of CO$_2$/HCO$_3$$_{-}$-independent acid-loading mechanisms not directly resulting from the generation of acid equivalents by cell metabolism has been explicitly taken into account while studying pH$_i$ regulation (7). Therefore, the mechanistic basis(es) for acid loading is currently very poorly understood. In general, there at least three different ways that overall acid loading can be imagined to occur: 1) reverse operation of the NHE, 2) H$^+$ production via cellular metabolism, and 3) transmembrane transport of acid equivalents by transporters other than the NHE.

External Na$^+$ removal resulted in the acidification of both mock-infected and HCMV-infected cells, a result consistent with reverse operation of the NHE. However, because DEA did not slow down the rate of acid loading caused by external Na$^+$ removal, reverse Na$^+$/$H^+$ exchange does not appear to be a major mechanism of acid loading in our studies.

Activity of DEA-insensitive proton-equivalent transporters as well as H$^+$ production would be expected to continue in the presence of DEA. We show DEA-insensitive acid loading in both mock-infected and HCMV-infected cells (Fig. 5), but HCMV infection reduces that rate. There is no direct evidence regarding the effect of HCMV infection on H$^+$ production via cellular metabolism. However, it has been reported

<table>
<thead>
<tr>
<th>Table 1. Effect of HCMV infection on cell properties</th>
<th>Mock Infected</th>
<th>HCMV Infected</th>
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<tr>
<td>Volume, pl (urea space)</td>
<td>1.47</td>
<td>6.16</td>
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<tr>
<td>Surface area, sites/cell (ouabain binding)</td>
<td>$1.07 \times 10^6$</td>
<td>$3.13 \times 10^6$</td>
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<tr>
<td>Buffering power, mM/pH unit</td>
<td>11.9 ± 2.4 (6)</td>
<td>6.9 ± 0.4 (11)</td>
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<tr>
<td>Flux via NHE, $H^+\cdot s^{-1}\cdot ouabain$-binding site$^{-1}$</td>
<td>38 ± 6 (4)</td>
<td>62 ± 7 (4)</td>
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<tr>
<td>Flux via acid loaders, $H^+\cdot s^{-1}\cdot ouabain$-binding site$^{-1}$</td>
<td>16 ± 2 (4)</td>
<td>3.5 ± 0.2 (4)</td>
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Values are means ± SE. Numbers of each kind of experiment are indicated in parentheses. Volume and surface area are from Altamirano et al. (3). NHE, Na$^+$/H$^+$ exchanger; HCMV, human cytomegalovirus. *Determined at intracellular pH (pH$_i$) = 7.45.
that glucose uptake has increased over sixfold when measured 54 h PE (27). This, coupled with the HCMV-induced increase of protein synthesis (38), would suggest that HCMV stimulates rather than reduces the cellular metabolism. Therefore, it seems unlikely that the reduced acid-loading rate observed for HCMV-infected cells is due to reduced metabolic production of H⁺.

We are left with the possibility that HCMV infection reduces other acid-loading ion transport mechanisms. In this regard, it is important to note that the negative intracellular resting membrane potential favors the net uptake of protons, perhaps by H⁺ channels (e.g., Ref. 28) or by loss of OH⁻ or HCO₃⁻. Some of our present results are consistent with a significant voltage-sensitive mechanism for acid loading. Replacement of extracellular Na⁺ with the impermeant cation NMDG⁺ not only inhibits the NHE, but would also be expected to increase the intracellular negativity of the resting membrane potential, thereby increasing the inwardly directed driving force on H⁺. In fact, we observed adification of both cell types on removal of external Na⁺. However, the rate was much faster for the mock-infected cells (Fig. 2) than for the HCMV-infected cells. It has been reported that HCMV-infected cells have a greatly reduced Na⁺ current (4) and would therefore be expected to hyperpolarize much less than mock-infected cells after external Na⁺ replacement. Thus the observed lower acidification rate of the HCMV-infected cells on external Na⁺ removal may be the result of the reduced driving force on H⁺ caused by a reduced hyperpolarization.

HCMV infection and cell volume. Altamirano et al. (3) showed that HCMV infection increased the cell water space (using [¹⁴C]urea) by as much as fourfold (see Table 1). An increase in cell volume is a hallmark of productive HCMV infection. In fact, without the host cell volume increase, the efficiency of viral replication is greatly reduced (see below). Cell volume is a fundamental cellular property, ordinarily under rather tight regulatory control in the resting, nondividing cell (19). In principle, the HCMV-induced cell enlargement could be the result of pathological effects on cell volume maintenance processes.

The present work focuses on the NHE because previous results linked the activity of Na⁺ transporters to the development of cytomegaly and efficient viral replication (31). Enhanced activity of the NHE coupled with activity of Cl⁻/HCO₃⁻ exchange provides the net osmo-lyte uptake by which cells bathed in CO₂/HCO₃⁻-containing media respond to cell shrinkage and regain their original volume. As a result of the concerted action of these two exchangers, Na⁺ and Cl⁻ plus osmotically obliged water enter the cell. Our present results are consistent with this same overall process playing a role in the initiation of the development of cytomegaly. Because most of the Na⁺ that enters will be exchanged for K⁺ by the sodium pump, this mechanism would also stimulate sodium pump activity, as has been previously shown by our group (3, 31). In the present work we add to the preceding circumstantial evidence for an Na⁺-dependent mechanism in cytomegaly by demonstrating an increase of NHE-mediated flux (Table 1).

The NHE in uninfected fibroblasts is stimulated by exposure to hyperosmotic solutions (e.g., Ref. 29; see Figs. 7 and 8). This osmosensitivity of the NHE presumably represents part of the normal cell volume maintenance mechanism. HCMV infection greatly enhanced the NHE osmosensitivity of MRC-5 cells (Fig. 8), a finding consistent with the view that HCMV-induced cytomegaly might be mediated, at least in part, by the NHE.

There are several possibilities to explain such a change in the osmosensitivity of the NHE. Other laboratories have reported that Cl⁻ affects the volume sensitivity of the NHE (15, 32, 33). We have reported preliminary evidence that two treatments expected to lower intracellular [Cl⁻] and alter its permeability will cause mock-infected cells to exhibit a more pronounced response to moderate hyperosmotic stimulation of the NHE (12, 14). Another variable reported to affect the volume sensitivity of the NHE is the intracellular protein concentration (9). There is evidence that the protein compositions of the mock-infected and HCMV-infected cells are different (38). The difference in protein composition might also contribute to our observed difference in cytoplasmic buffering power. Thus it is possible that the osmosensitivity of mock-infected and HCMV-infected cells differ significantly with regard to one or both of these variables.

Also there is the possibility that HCMV may affect the microenvironment of the NHE. In a study of SV40-transformed MRC-5 fibroblasts, a decrease in membrane cholesterol apparently enhanced hyperosmotic stimulation of the NHE, whereas enrichment apparently inhibited almost all osmotically induced activity (30). Therefore, HCMV-induced structural changes in the lipid bilayer in which the NHE is incorporated may account, at least in part, for the observed differences between HCMV-infected and mock-infected cells.

Mitogenic-like effects of HCMV. Mitogens both cause an alkaline shift in the pH, dependence of NHE (e.g., Ref. 18) and inhibit acid-loading mechanisms (7). In addition, phorbol ester treatment has been shown to increase both Vmax and K0.5(Na), for the NHE (10). These are similar results to those we have reported in the present work to be caused by HCMV infection. Thus the present results add to the list of HCMV-induced effects that mimic mitogenic cell activation (1). It is tempting to speculate that increased NHE activity in mitogenically stimulated cells and HCMV-infected cells serves an important role in the development of cell volume increases in both conditions.

The change in Vmax and K0.5(Na), (Fig. 6) might possibly be due to an isoforome change. However, increased Na⁺/H⁺ exchange activity observed in SV40-transformed MRC-5 cells has been reported to be the result of an increase in the turnover rate of the native NHE1 isoforome of the MRC-5 cells (35). In addition,
changes of the $K_{0.5}(\text{Na})$ as a result of cell maturation have been reported for HL-60 cells (11). The alkaline shift in the pH, dependence that we report (Fig. 5) is similar to that reported to be due to the long-term expression of Ga13 (40) of H-ras (24). Thus, although our data do not permit us to rule out an isomorph change, equally likely explanations include any or all of the following: the expression of cellular and/or HCMV “regulatory” proteins, posttranslational modifications of NHE1, and/or an altered microenvironment for the NHE. Finally, because a variety of host cells infected with HCMV will synthesize and secrete cytokines (37), it is possible that the effect we have shown is the result of cytokine stimulation of the NHE (5). Much more work will be needed to determine the basis(es) of the effects we have reported. Regardless of the mechanism(s) involved, we have clearly shown that HCMV has dramatic effects on the functional properties of the NHE.

Viral replication and host cell microenvironment. The development of cytomegaly by the host cell and efficient viral replication are closely related phenomena, and Na+ entry into the host cell appears to be closely involved in this relationship. Byun et al. (31) have shown that bathing HCMV-infected cells in a low-[Na+] medium inhibits cell enlargement, reduces Na+ pump stimulation, and greatly reduces viral replication rate. Altamirano et al. (3) showed that exposure to low [Na+] reduced the increase in the number of ouabain-binding sites caused by HCMV infection. Fons et al. (17) showed that 150 $\mu$M amiloride, an inhibitor of the NHE, arrested the development of cytomegaly and reduced viral yields by 100-fold. It is unknown whether it is the increase in cell volume, changes in the intracellular ion composition, or both, that are required for cytomegaly and efficient viral replication. It is possible that alterations of the ionic composition of cytoplasm, particularly sodium, may differentially affect viral and host cell protein synthesis because it is known that the HCMV-encoded DNA polymerase has a different salt sensitivity than the host cell polymerase (23). Alterations in inorganic ion balance caused by the virus and differences in the salt sensitivity between the two polymerases may account, at least in part, for the shift from host cell protein synthesis to that of viral protein synthesis that occurs during the first 48 h PE (38).

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