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Rapid Increase in pH Set-Point of the Na^+-*in*-dependent Chloride/Bicarbonate Antiporter in Vero Cells Exposed to Heat Shock

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Abstract. Internal pH (pH_i) is in Vero cells regulated mainly by three antiports. Na^+/H^+ antiport and Na^+-dependent Cl^-/HCO_3^- antiport increase pH_i in acidified cells, and Na^+-*in*dependent Cl^-/HCO_3^- antiport lowers pH_i in cells after alkalinization. The activities of the antiporters were altered in cells after exposure to 41–45°C. Under such conditions the Na^+/H^+ antiport and the Na^+-dependent Cl^-/HCO_3^- antiport were both stimulated, whereas the Na^+-*in*dependent Cl^-/HCO_3^- antiport was inhibited in such a way that a higher pH value was required to activate it. This alteration was also induced by some other forms of cellular stress, but did most likely not involve stress proteins as protein synthesis was not required. The possibility of regulation by alteration in protein phosphorylation is discussed.

Key words: Anion antiport — pH regulation — HCO_3^- — Heat shock — Hyperthermia

Introduction

Different tissues and cells exhibit different sensitivities to heat (for review, *see* Calderwood & Dickson, 1983). This finding has been utilized in cancer therapy as some tumors are damaged at temperatures tolerated by normal tissues (Cavaliere et al., 1967; Dickson & Shah, 1977). The molecular mechanism for cell killing by moderate heat is not understood, but several in vitro studies demonstrate that at low pH cells are more easily damaged by heat than at higher pH values (Overgaard, 1976; Freeman, Dewey & Hopwood, 1977; Gerweck, 1977; Hofer & Mivechi, 1980; Gerweck & Richards, 1981). Current evidence indicates that the intracellular pH (pH_i) rather than the pH in the medium (pH_o) is decisive for the sensitivity to heat (Haveman, 1979; Hofer & Mivechi, 1980; Chu & Dewey, 1988; Lyons, Kim & Song, 1992).

The most conspicuous alteration in cells exposed to increased temperature is a sharp reduction in normal protein synthesis, followed by induction of synthesis of a small group of proteins known as heat shock proteins (HSP) or stress proteins (Craig, 1985; Lindquist, 1986; Subjeck & Shyy, 1986). Increased levels of mRNAs encoding HSP can be detected in the cytoplasm of eukaryotic cells approximately 30 min after a temperature increase. However, before this event, only minutes after the heat shock is given, changes are observed in the pattern of protein phosphorylation (Chrétien & Landry, 1988; Landry et al., 1989), suggesting that heat shock changes protein kinase or phosphoprotein phosphatase activities.

Altered protein phosphorylation and induction of HSP are thought to increase the ability of the cells to survive at the high temperature. In fact, cells that have been given a sublethal heat shock are for hours more resistant to a subsequent heat shock than control cells (Li & Werb, 1982; Subjeck & Shyy, 1986; Landry, Lamarche & Chrétien, 1987; Créte & Landry, 1990). The mechanism for induction of tolerance is not clear, but a candidate key protein for tolerance induction is HSP 27. Increased levels of this protein are induced by heat shock, and transfection studies have shown that HSP 27 is sufficient to confer a thermoresistant phenotype upon Chinese hamster O23 cells (Chrétien & Landry, 1988; Landry et al., 1989). Intriguingly, however, also in the absence of HSP induction and, even in the absence of protein synthesis, cells appear to become thermoresistant (Li & Werb, 1982; Landry et al., 1987; Lee, Dewey & Li, 1987; Laszlo, 1988). Two kinds of thermotolerance have been proposed—one that is dependent on HSP synthesis and

one that is not. Créte and Landry recently showed (1990) that phosphorylation of a pre-existing pool of HSP 27 coincided with heat resistance in O23 cells.

Cells in which the cytoplasm is acidified prior to heat shock do not acquire thermotolerance and die within a short period of time at the elevated temperature (Freeman et al., 1980; Cook & Fox, 1988*a*). However, if the cells are adapted to growth at low pH_o before heat treatment, they may develop thermotolerance. Cells adapted to low pH_o were shown to have higher pH_i than control cells exposed to the same pH_o (Chu & Dewey, 1988; Cook & Fox, 1988*a*). Also, cells adapted to elevated temperatures were more capable of dealing with environmental pH changes than control cells (Hahn, 1986; Hahn & Shiu, 1986).

In spite of the great current interest in the heat shock response, the effect of heat shock on the different mechanisms that regulate pH_i has been studied only to a limited extent (Kiang, McKinney & Gallin, 1990; Wang et al., 1990). In eukaryotic cells pH_i is regulated within narrow boundaries. If H^+ ions were passively distributed over the cell membrane, the pH_i would be ~1 pH unit below neutrality and incompatible with life (Roos & Boron, 1981). Cells are therefore equipped with mechanisms to counteract acidification either by export of H^+ or import of HCO_3^-. The best studied mechanism to increase pH_i is the Na^+/H^+ antiport which uses the large inwardly directed Na^+ gradient for proton extrusion (Moolenaar, 1986*a*). This antiport is present in most eukaryotic cells and is probably the most important mechanism to counterbalance severe acid loads. The Na^+/H^+ antiporter is regulated by pH_i as well as by a number of hormones and other ligands binding to cells (Frelin, Vigne & Lazdunski, 1983; Grinstein, Rothstein & Cohen, 1985; Moolenaar, 1986*b*). Many cells also possess a Na^+-linked Cl^-/HCO_3^- antiporter which exchanges Na^+ and HCO_3^- for Cl^- and thereby alkalinizes the cytosol. This antiport has been shown to be constitutively active in several cell lines. It plays an important role in regulating pH_i after small deviations to the acidic side (Tønnessen, Sandvig & Olsnes, 1990).

A Na^+-*in*dependent Cl^-/HCO_3^- antiport reduces pH_i in cells that have obtained an alkali load. This antiport is regulated to different extents in different cell lines primarily by pH_i but also by serum and by a number of drugs (Tønnessen et al., 1989*a,b*; Tønnessen et al., 1990). When it is activated, the inwardly directed Cl^- gradient extrudes HCO_3^- from the cells by antiport and thereby reduces pH_i. Under steady-state conditions pH_i is apparently maintained by the balance between the alkalinizing Na^+-linked Cl^-/HCO_3^- antiport and the acidifying Na^+-*in*dependent Cl^-/HCO_3^- antiport (Tønnessen et al., 1989*a*; Olsnes & Sandvig, 1986).

The Na^+-*in*dependent Cl^-/HCO_3^- antiport is particularly interesting because in many cells it is strongly regulated by pH_i. In the monkey kidney cells, Vero, the activity of the antiport is increased 5–10 fold when pH_i is raised 0.2–0.3 pH units (Olsnes, Tønnessen & Sandvig, 1986). This sharp regulation could involve seven or more cooperative allosteric binding sites for H^+ (Ludt et al., 1991). Phosphorylations could be involved in the regulation of this antiporter, as both stimulation and down regulation of protein kinase C by short-term and long-term treatment with TPA, respectively, have strong effects on the pH_i sensitivity of the antiport (Ludt et al., 1991). In the present work we have investigated the effect of heat shock on the modulation of the different pH-regulatory mechanisms in Vero cells.

Materials and Methods

Cells

Vero cells (from African green monkey kidney) were grown in DMEM (pH 7.4) with 5% fetal calf serum in air containing 5% CO_2. For ion-flux experiments cells were seeded out into 24-well disposable trays (10^5 cells/well) in medium containing 10% fetal calf serum two days before use. For the purpose of measuring pH_i by fluorometry, cells (2×10^5 cells/cm^2) were seeded out in the same medium the day before use on coverslips placed in 5-cm petri dishes.

Chemicals

MES (2-(N-morpholino)ethane sulfonic acid, Tris (tris(hydroxymethyl)aminomethane), potassium gluconate, nigericin, HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), DIDS (4,4'-diisothiocyanostilbene-2,2'-disulfonate), choline-bicarbonate and ouabain were obtained from Sigma. $H^{36}Cl$ (specific activity 19.6 μCi/mg chloride) and $^{22}NaCl$ were from The Radiochemical Centre, Amersham, UK, TCA (trichloroacetic acid) was from Merck (Darmstadt, FRG) and amiloride was generously given to us by Merck, Sharp & Dohme (Drammen, Norway).

Buffers

Mannitol buffer (mM): 260 mannitol, 1 $Ca(OH)_2$, 20 MES adjusted to the indicated pH with Tris. Potassium gluconate buffer (mM): 140 potassium gluconate, 1 $Ca(OH)_2$, 20 HEPES adjusted to the indicated pH with Tris. Phosphate buffered saline (PBS) (mM): 140 NaCl and 10 sodium phosphate, pH 7.4. KCl buffer (mM): 140 KCl, 1 $CaCl_2$, 20 MES adjusted to the indicated pH with Tris. NaCl buffer (mM): 140 NaCl, 1 $CaCl_2$, 20 MES adjusted to the indicated pH with Tris. HEPES medium: DMEM buffered with 20 mM HEPES instead of bicarbonate.

Measurement of $^{36}Cl^-$ Uptake

To measure uptake, cells in 24-well microtiter trays were preincubated as indicated and then washed twice in ice-cold mannitol buffer, pH 7.0. Mannitol buffer (0.3 ml) or potassium gluconate buffer containing 0.17 μCi (0.5 mM) of $H^{36}Cl$ was then added per well. The cells were incubated at 24°C for the indicated time, then rapidly washed twice with ice-cold PBS, and finally 0.3 ml per well of 5% (w/v) trichloroacetic acid was added to extract the radioactivity. After 10 min at room temperature, the TCA was transferred to counting vials and the radioactivity was measured. In some cases the cells remaining in the plastic wells were subsequently dissolved in 0.2 M KOH, the absorption at 280 nm was measured and the number of cells was estimated from a calibration curve.

Measurement of $^{22}Na^+$ Uptake

As indicated in the figure legends, after preincubation cells in 24-well microtiter trays were transferred to mannitol buffer, pH 7.4, containing 1 mM amiloride, 50 μM ouabain, 20 mM choline bicarbonate and 0.8 μCi $^{22}NaCl$ and incubated for 4 min at room temperature. The escape of CO_2 from the medium during this short period was insignificant. The buffer was then removed by suction, each well was washed three times with PBS, and 0.3 ml of 5% TCA was added. The radioactivity associated with the cells was measured (Olsnes et al., 1987).

Measurement of pH_i

For continuously monitoring of pH_i, the fluorescent probe BCECF was used. The cells were seeded out on glass coverslips (1 × 3 cm) and grown to confluency. They were then incubated with BCECF (2–5 μM) in HEPES-medium without pH indicator at 37°C for 15–45 min. The coverslip was inserted into the cuvette which was then placed in a thermoregulated chamber in a Perkin Elmer LS-50 spectrofluorimeter at an angle of 30° to the excitation source. Excitation wavelengths of 450 and 500 nm and emission wavelength of 530 nm were used, and the slits were adjusted to yield an intensity of about 800 at 500 nm excitation at the start of the experiment. Buffers were changed by perfusion. Calibration curves were obtained from experiments where the internal pH was clamped by incubation in isotonic KCl with 10 μM of the ionophore nigericin, and pH_i was calculated from the intensity ratios between the two excitation wavelengths.

Abbreviations

Tris (tris(hydroxymethyl)aminomethane); HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid); DIDS (4,4'-diisothiocyanostilbene-2,2'-disulfonate); TPA (12-O-tetradecanoylphorbol 13-acetate); MES 2-(N-morpholino)ethane sulfonic acid; DMEM (Dulbecco's modified minimum essential medium); PBS (phosphate buffered saline); pH_i (intracellular pH); pH_o (extracellular pH); TCA (trichloroacetic acid); BCECF (2',7'-bis[carboxyethyl]-5,6-carboxyfluorescein).

Results

Effect of Heat Shock on Chloride Self-Exchange

When cells are transferred from growth medium containing 120 mM Cl^- to a Cl^--free buffer osmotically balanced with mannitol or gluconate and containing trace amounts of $^{36}Cl^-$, $^{36}Cl^-$ is accumulated in the cells by antiport energetically driven by Cl^- efflux. We have earlier presented evidence that in Vero cells this Cl^-/Cl^- self-exchange is mainly carried out by the Na^+-*in*dependent Cl^-/HCO_3^- antiporter and that the rate of chloride self-exchange is a measure for the activity of this antiporter (Olsnes & Sandvig, 1986; Olsnes et al., 1986; Tønnessen et al., 1987).

To test the effect of heat shock on chloride self-exchange, cells were incubated for 15 min at 45°C and then for 15 min at 37°C in medium adjusted to various pH values. Subsequently, the cells were transferred to mannitol-balanced buffer containing a trace (0.5 mM) $^{36}Cl^-$ and the uptake was measured at 24°C. Control cells were treated in identical manners, except that they did not receive the heat shock. As Fig. 1*A* shows, the uptake of $^{36}Cl^-$ was reduced after heat shock at all the pH_o values tested, but the effect was most pronounced when the cells were preincubated at pH_o 6.8–7.0. Five minutes preincubation at pH_o 6.8 and 45°C was sufficient to induce a substantial reduction in $^{36}Cl^-$ uptake (Fig. 1*B*). Even when the heat shock lasted for 45 min the inhibitory effect was essentially the same (*not demonstrated*).

In the experiments above the cells were incubated at 37°C for 15 min after the heat shock before $^{36}Cl^-$ uptake was measured. This was done to assure that there was no direct temperature effect on the uptake. The data in Fig. 1*C* show that the uptake was essentially the same whether the incubation at 37°C was for 8 or 15 min. On the other hand, after 30 min the inhibitory effect was reduced. This indicates that the inhibitory effect is rapidly induced by heat shock and somewhat more slowly reversed at 37°C. In all cases the $^{36}Cl^-$ uptake was strongly inhibited by DIDS which is a potent inhibitor of anion antiport (*data not shown*).

In general, pH_i increases with increasing pH_o, and we have shown earlier that the rate of $^{36}Cl^-/Cl^-$-exchange is dependent on pH_i (Olsnes et al., 1986). However, it is difficult to manipulate pH_i in a controlled manner by varying pH_o. To test the effect of heat shock on the pH sensitivity of the antiport, we therefore clamped the pH_i by preincubating the cells in buffer containing nigericin and

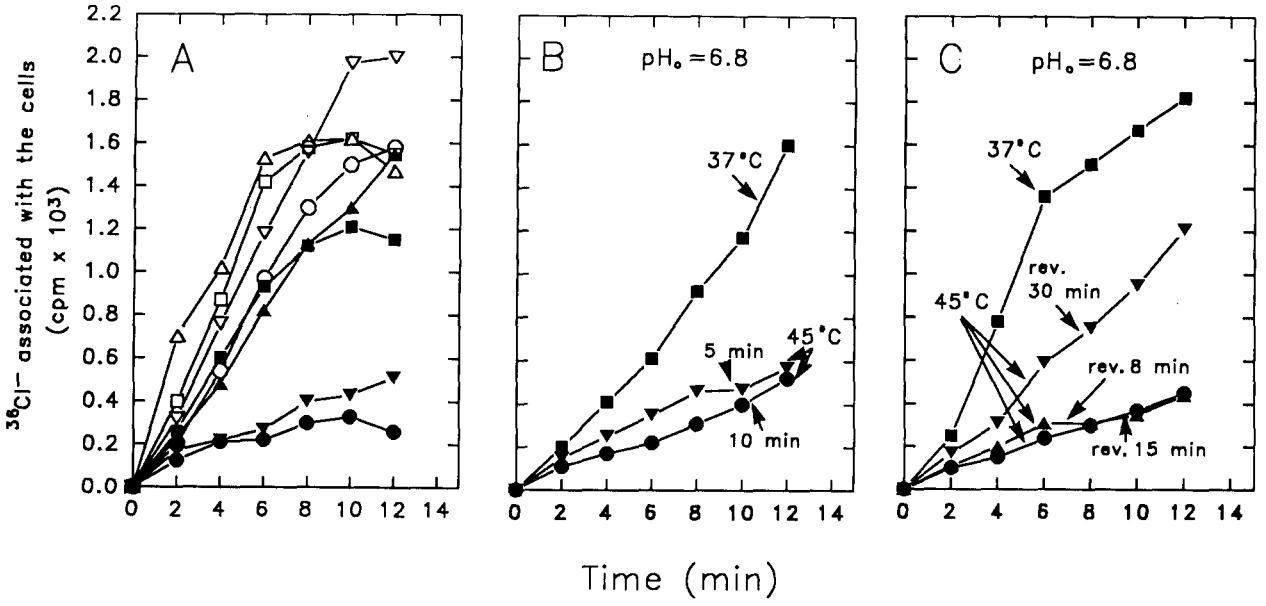

Fig. 1. Effect of heat shock on uptake of $^{36}Cl^-$ in Vero cells. (*A*) Cells were incubated for 15 min in HEPES medium, pH 6.8 (○, ●), 7.0 (▽, ▼), 7.2 (□, ■) or 7.4 (△, ▲) at 37°C (open symbols) or 45°C (filled symbols) and then transferred to HEPES medium, 37°C, with the same pH. After 15 min the medium was removed, the cells were washed twice in ice-cold mannitol buffer, pH 7.0, and then the uptake of $^{36}Cl^-$ was measured in the same buffer at room temperature for the indicated times. In (*B*) the preincubation was at pH 7.4, and the cells were then transferred to HEPES medium, pH 6.8, at 37°C for 8 min (■) or at 45°C for 5 min (▼) or 10 min (■). The uptake of $^{36}Cl^-$ was then measured as in *A*. In (*C*) the preincubation was at pH 6.8 for 15 min at 37 or 45°C. To test the reversal rate, the cells were then incubated in the same medium at 37°C for 8 min (▲), 15 min (●) or 30 min (▼) before the uptake of $^{36}Cl^-$ was measured as in *A* and *B*.

isotonic KCl. When the extracellular and intracellular concentrations of K^+ are identical, the K^+/H^+ ionophore, nigericin, equilibrates the external and internal pH, and pH_i can therefore be manipulated by varying the pH in the external buffer (Thomas et al., 1979). Cells preincubated at 37 or 45°C were pH-clamped for 15 min at different pH values and then transferred to K-gluconate buffer adjusted to the same pH, and containing 2 μM nigericin and 0.5 mM $^{36}Cl^-$. The amount of radioactivity associated with the cells was measured after various times. The results in Fig. 2*A* show that while there was little difference whether or not the cells had been exposed to heat shock when pH_i was clamped at pH 6.8 and 7.0, the rate of $^{36}Cl^-$ accumulation was reduced in the heat-shocked cells when the uptake was measured at pH 7.2 and 7.4.

To obtain a more detailed picture of the effect of heat shock on the pH sensitivity of the $^{36}Cl^-/Cl^-$ exchange, heat-shocked and control cells were incubated with KCl-nigericin at a number of different pH values and then the amount of $^{36}Cl^-$ uptake during 2.5 min was measured. As shown in Fig. 2*B*, the uptake increased strongly with increasing pH during the preincubation, in accordance with our earlier findings (Olsnes & Sandvig, 1986; Olsnes et al., 1986; Tønnessen et al., 1989*a*; Ludt et al., 1991). The uptake was strongly inhibited by DIDS. The important finding here is that after preincubation at 44–45°C for 15 min, half-maximal activation of the antiport was obtained at higher pH values than in the control cells. The difference was found to be 0.15 ± 0.05 pH units in 17 independent experiments. The same effect was obtained when the heat shock was given by incubating cells for 3 hr at 41°C. There was no further increase in the activation pH when the incubation time at 45°C was 30 rather than 15 min (*data not shown*). Altogether, the data indicate that a moderate heat shock rapidly increases the set-point of the Na^+-*in*dependent Cl^-/HCO_3^- antiport by ~0.15 pH units.

As Cl^- efflux is the driving force for $^{36}Cl^-$ uptake in these experiments, it was essential to test if the chloride content of the control cells was different from that of the cells that had been incubated at 45°C. For this purpose, we loaded the cells with $^{36}Cl^-$ at 37°C for 3 hr in HEPES medium, pH 7.4, containing 1.7 μCi $^{36}Cl^-$. Some of the cells were then transferred to 45°C for 15 min, while the control cells remained at 37°C. The cells were then rapidly chilled to 0°C, washed, and the remaining radioactivity extracted. As the Table shows, there was no difference in the chloride content of the cells under the two conditions.

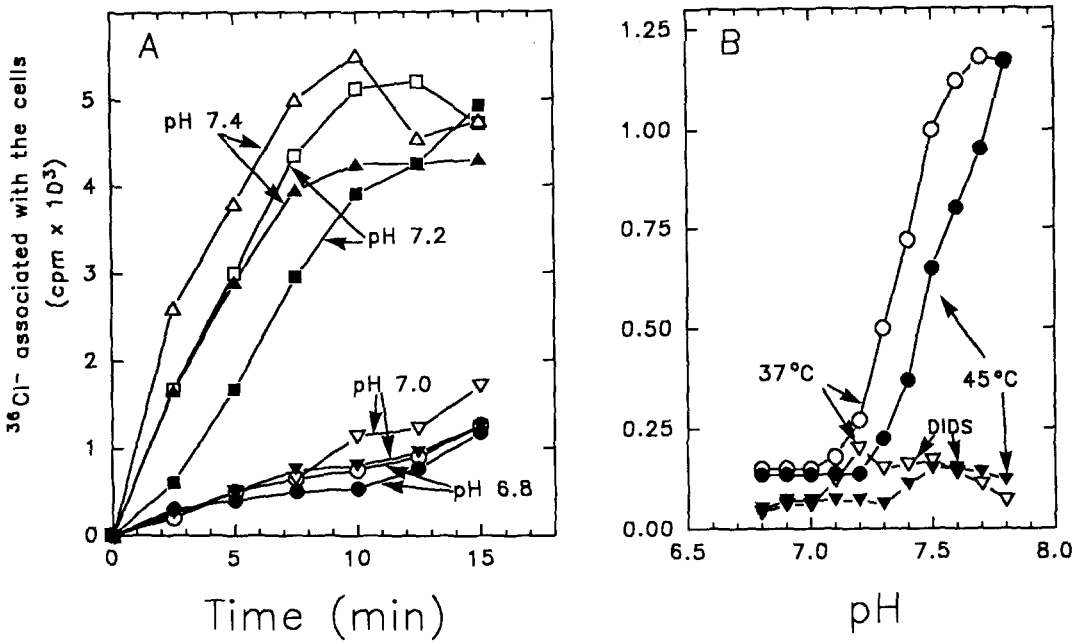

Fig. 2. Effect of heat shock on $^{36}Cl^-$-uptake in pH_i-clamped cells. (*A*) Cells were preincubated for 15 min in HEPES medium, pH 7.4, at 37°C. In some cases they were then transferred to the same medium, adjusted to 45°C (filled symbols), whereas other cells were kept at 37°C (open symbols). After 15 min, the cells were transferred to KCl-buffer containing 2 μM nigericin, adjusted to pH 6.8 (○, ●), 7.0 (▽, ▼), 7.2 (□, ■) or 7.4 (△, ▲). After 15 min at 37°C the cells were transferred to the same buffer except that it contained 0.17 μCi/ml $^{36}Cl^-$ and the uptake was measured after the indicated times. In (*B*) conditions were the same as in *A* except that the KCl buffer was adjusted to a number of pH values, as indicated on the abscissa, and the $^{36}Cl^-$ uptake was measured in mannitol buffer during 2.5 min. When indicated, 1 mM DIDS was present during the pH clamping and uptake period. The average difference in half-maximal uptake rate was 0.15 ± 0.05 pH units (n = 17).

Table. Effect of hyperthermia on the chloride content of the cells

Experimental condition	Temperature	
	37°C	45°C
$^{36}Cl^-$ (cpm)	747 ± 28	747 ± 62

The cells were incubated for 3 hr at 37°C in HEPES medium, pH 7.4, containing 1.7 μCi $^{36}Cl^-$. Some of the cells were then transferred to 45°C, while the control cells were maintained at 37°C and the incubation was continued for 15 min. The cells were subsequently chilled to 0°C, rapidly washed with ice-cold PBS, and the TCA-soluble radioactivity was measured. The data represent mean ± SD in five experiments.

It was also conceivable that the rate of leakage of Cl^- in the absence of extracellular Cl^- was altered after heat shock, and that the chloride content under the experimental conditions used in Fig. 2 was therefore different. There is, in Vero cells, a certain uncoupled (electrogenic) efflux of chloride into buffers containing no permeant anions. This efflux strongly increases with increasing pH_i (Olsnes et al., 1986). To see if the efflux into mannitol buffer was altered after heat shock, cells were first preincubated at pH 6.0 to slow down the efflux of Cl^- before loading the cells with $^{36}Cl^-$. The cells were then loaded with $^{36}Cl^-$ for 30 min in mannitol buffer (pH 6.0) containing $^{36}Cl^-$. During the last 15 min of this period, the temperature was raised to 45°C in some cases. The cells were then transferred to K-gluconate buffer adjusted to different pH values and containing nigericin to equilibrate pH_i with that in the buffer and allow $^{36}Cl^-$ efflux to take place. After 15 min the amount of $^{36}Cl^-$ remaining in the cells was extracted and counted. As Fig. 3 shows, the efflux rate increased strongly with pH both in control (half-maximal at pH 7.05) and heat-shocked cells. In the heat-shocked cells, however, the efflux curve was slightly shifted to higher pH values (half-maximal rate at pH 7.15). The reduced uptake rate by anion antiport in the heat-shocked cells in Fig. 2, therefore, cannot be accounted for by reduced chloride content in the cells.

Effect of Heat Shock on Na^+/H^+ Antiport and on Na^+-Dependent Cl^-/HCO_3^- Antiport

Inhibition of Na^+-*in*dependent Cl^-/HCO_3^- antiport as seen after heat shock would tend to increase pH_i and thereby protect the cells against elevated tem-

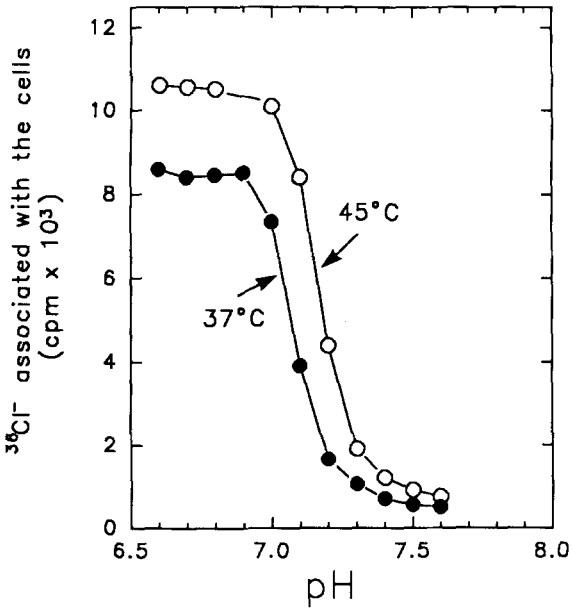

Fig. 3. Effect of heat shock on $^{36}Cl^-$ efflux in pH_i-clamped cells. The cells were incubated at 37°C in HEPES medium, pH 6, for 30 min and then transferred to mannitol buffer, pH 6, containing 0.17 μCi (0.5 mM) $^{36}Cl^-$. After 15 min some of the cells (○) were transferred to 45°C while the control cells remained at 37°C (●) and the incubation was continued for another 15 min. The cells were then washed twice in ice-cold mannitol buffer, and K-gluconate buffer, pH indicated on the abscissa, containing 2 μM nigericin was added at 37°C. After 15 min the cells were washed and TCA precipitated.

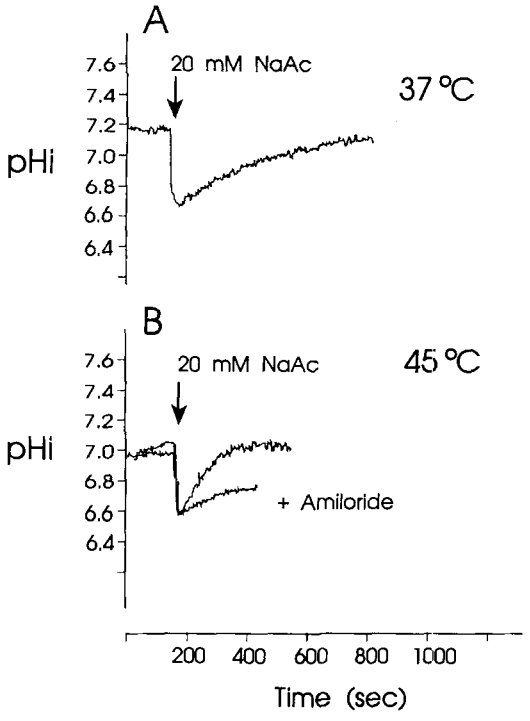

Fig. 4. Effect of heat shock on pH_i in the absence of HCO_3^-. Cells were incubated for ~30 min in HEPES medium (without phenol red), pH 7.2, in the presence of 3 μM BCECF. They were then transferred to NaCl buffer with the same pH at either 37°C (*A*) or 45°C (*B*) and the incubation was continued for 15 min more. The ratio between the 530 nm emission obtained at excitation wavelengths of 450 and 500 nm was then monitored continuously at 37°C. After approximately 150 sec a final concentration of 20 mM sodium acetate (pH 7.2) was added. When indicated 1 mM amiloride was present from time zero of the monitoring. The data are representative for five independent experiments.

perature. To test if other pH-regulatory mechanisms were also modified, we tested the effect of heat shock on Na^+/H^+ exchange and on Na^+-coupled Cl^-/HCO_3^- antiport which both tend to increase pH_i.

The activity of the amiloride sensitive Na^+/H^+ antiport can be measured by monitoring pH_i recovery after acidification of the cytosol. The antiport was activated by a cytosolic acid load, and the activity of the Na^+/H^+ antiport was measured in the absence of bicarbonate to avoid the influence of the Na^+-dependent Cl^-/HCO_3^- antiport. Cells were loaded with the pH-sensitive dye BCECF, part of them was given heat shock (15 min at 45°C), whereas another part was kept at 37°C as control. pH_i was then monitored at 37°C. At the start of the experiment, pH_i was in the control cells 7.2 (Fig. 4*A*), while it was 7.0 in the heat-shocked cells (Fig. 4*B*). After 3 min the cytosol was acidified by the addition of 20 mM sodium acetate (pH 7.2). The protonated form of the acid enters the cytosol by passive diffusion through the cell membrane and then dissociates and acidifies the cytosol. In the control cells, pH_i recovery occurred slowly (Fig. 4*A*), and the initial pH_i was recovered only after approximately 500 sec. After the cells had been heat shocked, however, the initial pH_i was recovered after ~200 sec (Fig. 4*B*). In the presence of the inhibitor of Na^+/H^+ antiport, amiloride, pH_i recovery was considerably inhibited.

The DIDS-sensitive Na^+-coupled Cl^-/HCO_3^- exchange can be monitored by measuring the transport of $^{22}Na^+$ into cells in the presence of HCO_3^- and amiloride. The activity of this antiport is not regulated by pH_i in Vero cells (Olsnes et al., 1987). To test the effect of heat shock on the antiport, cells were incubated at 45 or 37°C for 15 min and then transferred to KCl buffer with nigericin adjusted to various pH values. Subsequently, we measured the uptake of $^{22}Na^+$ in mannitol buffer containing 20 mM choline bicarbonate, in order to generate an outwardly directed chloride gradient to stimulate the uptake of ^{22}Na-HCO_3 by antiport. The buffer contained amiloride to inhibit the Na^+/H^+ exchanger. We have demonstrated previously that under these conditions $^{22}Na^+$ uptake by the Na^+-dependent Cl^-/HCO_3^- antiport can be measured selectively in

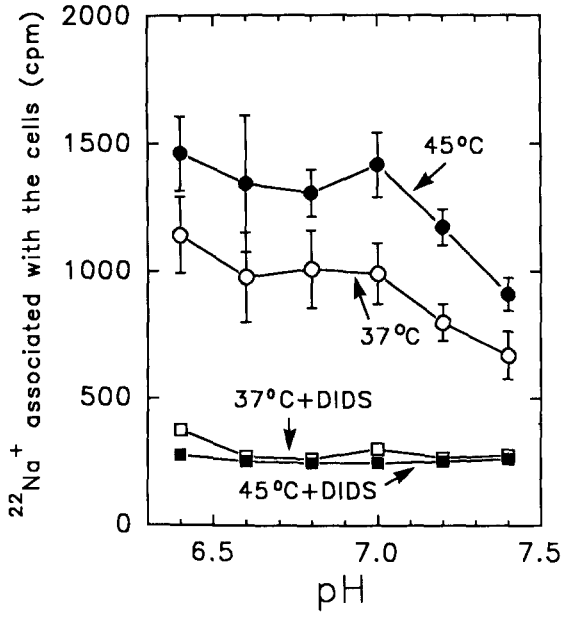

Fig. 5. Effect of hyperthermia on $^{22}Na^+$ uptake by Na^+-dependent Cl^-/HCO_3^- antiport. Cells were incubated in HEPES medium, pH 7.4, at 37°C for 30 min and the incubation was then continued for 15 min at 37°C (open symbols) or 45°C (filled symbols). The cells were transferred to KCl buffer with the pH indicated on the abscissa and containing 5 μM nigericin, 50 μM ouabain, 1 mM amiloride and when indicated 1 mM DIDS, and incubated for 10 min at 37°C. Mannitol buffer, pH 7.4, containing 1 mM amiloride, 50 μM ouabain, 0.8 μCi $^{22}Na^+$, 20 mM choline bicarbonate and when indicated 1 mM DIDS was then added, and the uptake of $^{22}Na^+$ during 4 min at room temperature was measured. The data represent mean ± SD in 10 experiments.

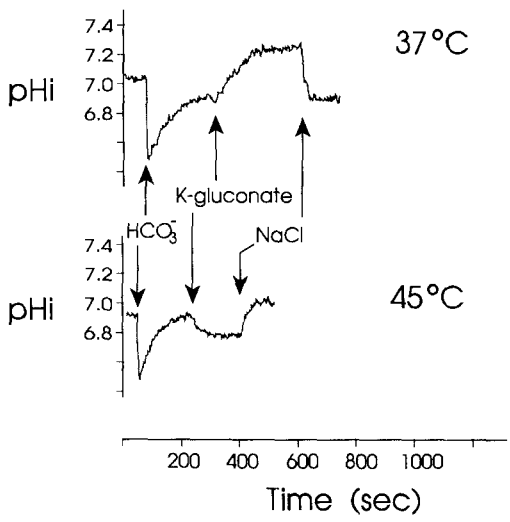

Fig. 6. Effect of heat shock on pH_i in the presence of bicarbonate. Cells were incubated in HEPES medium, pH 7.0, with BCECF for 30 min at 37°C. They were then transferred to NaCl buffer with the same pH at either 37 or 45°C and further incubated for 15 min. pH_i was monitored at 37°C in NaCl buffer, pH 7.0. After ~100 sec, 15 mM HCO_3^- was added and remained present for the rest of the experiment. When indicated, Na^+ and Cl^- were removed by addition of K-gluconate buffer with the same pH. Subsequently the K-gluconate buffer was removed and NaCl buffer was readded, as indicated. The data are representative for five experiments each.

Vero cells (Olsnes et al., 1987). As Fig. 5 shows, the antiport was somewhat stimulated at all the pH values tested after heat shock. The uptake could be inhibited by DIDS. Altogether, the results indicate that both of the two acid-extruding antiporters are stimulated after heat shock.

Effect of Heat Shock on pH_i

All three pH-regulatory mechanisms studied here were affected by heat shock in such a way as to increase pH_i. To study if pH_i did indeed increase, cells were loaded with BCECF, incubated at 37 or 45°C for 15 min, and finally pH_i was monitored fluorimetrically.

The results are shown in Fig. 6. The internal pH was first monitored in the absence of HCO_3^- at pH_o 7.0, and then HCO_3^- was added as indicated. In the absence of HCO_3^- pH_i was initially ~0.2 units lower in the heat-shocked cells (lower trace) than in the control cells (upper trace). Upon addition of HCO_3^- the cytoplasm was rapidly acidified by membrane-permeant CO_2 that enters the cytoplasm and reacts with water to form H_2CO_3, which then dissociates to H^+ and HCO_3^-. In both cases, pH_i immediately started to increase. The pH_i value achieved in the presence of HCO_3^- was the same whether or not the cells had been heat shocked. The result indicates that heat shock leads to acidification of the cytosol and that this effect is compensated for by the activities of the Na^+/H^+ and the Na^+-linked Cl^-/HCO_3^- antiports (Frelin et al., 1983; Grinstein, 1985; Olsnes et al., 1987).

In the same experiment we also measured the activity of the Na^+-*in*dependent Cl^-/HCO_3^- antiport by removing chloride from the extracellular medium in the absence of Na^+ (transfer to K-gluconate buffer). Under these conditions the outwardly directed Cl^- gradient pulls HCO_3^- into the cells by Cl^-/HCO_3^- antiport, with alkalinization of the cytosol as a result (Tønnessen et al., 1987). In accordance with earlier findings (Tønnessen et al., 1987), upon addition of K-gluconate buffer, pH_i in the control cells started to rise as a consequence of reversed Na^+-*in*dependent Cl^-/HCO_3^- exchange. In the heat-shocked cells there was no alkalinization upon Cl^- removal (addition of K-gluconate buffer), indicating that Na^+-*in*dependent Cl^-/HCO_3^- antiport was inactive. In fact, the cytosol was actually acidified after

removal of Na^+ and Cl^-, most probably due to reversal of the Na^+/H^+ exchanger which may be activated under these conditions (*see* Fig. 4). Actually, when 15 mM K^+ was replaced by Na^+ in the K^+-gluconate buffer to eliminate the outwardly directed Na^+-gradient, there was no acidification (*data not shown*). In all cases pH_i was rapidly restored by readdition of 0.14 M NaCl. Altogether, the data indicate that in heat-shocked cells there is increased acid production and that the three pH-regulatory antiporters are regulated to compensate for the increased acid load.

Effects of Induction and Inhibition of HSP Synthesis on the Regulation of Na^+-Independent Cl^-/HCO_3^- Antiport

In attempts to elucidate if the effects of heat shock on the pH regulation of the Na^+-*in*dependent Cl^-/HCO_3^- antiport is linked to HSP synthesis, we treated the cells with the known inducers of HSP; ethanol, 2-deoxyglucose, arsenite and the calcium-ionophore A-23187. The cells were then transferred to KCl/nigericin buffer containing the same compounds, and 15 min later the $^{36}Cl^-$ uptake during 2.5 min was measured in the same way as in Fig. 2*B*.

As shown in Fig. 7*A*, preincubation of the cells with 0.5% ethanol or with 10 mM 2-deoxyglucose for 2 hr before the pH clamping had no clear effect on the pH_i regulation of the antiport. Preincubation of the cells for 4 hr with 50 μM sodium arsenite shifted the curve to lower pH values (*data not shown*), whereas preincubation for 2 hr with 10 μM A-23187 shifted it by almost 0.2 pH units to higher pH values. Clearly, only treatment with the calcium-ionophore mimicked the effect of heat shock on the antiport. The effect was already evident 10 min after addition of the ionophore and it was dependent on extracellular calcium. The effects of A-23187 and heat shock appeared to be additive (*data not shown*).

To further investigate if HSP synthesis is required for the heat shock effect on the Na^+-*in*dependent Cl^-/HCO_3^- antiport, we inhibited protein synthesis with cycloheximide shortly before the heat shock and throughout the rest of the experiment. This treatment inhibited protein synthesis almost completely. As Fig. 7*B* shows, not only did cycloheximide not prevent the heat shock induced shift of the curve to higher pH values, but in the absence of heat shock it shifted the curve in a similar manner as heat shock. Cycloheximide and heat shock together seemed to have an additive effect. Puromycin had a similar effect as cycloheximide (*data not shown*). We may conclude that protein synthesis is not essential for the effect of heat shock on the pH regulation of Na^+-*in*dependent Cl^-/HCO_3^- antiport.

Discussion

The main finding in the present paper is that after exposure of Vero cells to heat shock, the activity of the Na^+-*in*dependent Cl^-/HCO_3^- antiport is inhibited. It should be noted that the effect was also detected after incubation at temperatures of physiological relevance (40–41°C), but at these temperatures longer exposure time was necessary than at 45°C. The data indicate that the Na^+-dependent pH-regulatory mechanisms, *viz.*, Na^+/H^+ antiport and Na^+-dependent Cl^-/HCO_3^- antiport, are stimulated by the same treatment. The effects observed were not due to kinetic alterations by temperature *per se*, as all the measurements that were compared were carried out at identical temperature.

In the case of the Na^+-*in*dependent Cl^-/HCO_3^- antiport the pH dependence was shifted in such a way that the pH required to activate the antiport was higher than in the control cells. This means that at a given pH around neutrality, the activity of the antiport was reduced after heat shock. On the other hand, at higher pH values there was little difference in the rate of uptake, demonstrating that heat shock did not induce a general reduction in $^{36}Cl^-$ uptake.

Three genes encoding cation-*in*dependent anion exchange have been cloned and sequenced, *viz.*, AE1 encoding band 3 in erythrocytes (Kopito, Anderson & Lodish, 1987), AE2 from a human erythroleukemic cell line (Demuth et al., 1986), murine preB lymphoblasts, mouse kidney (Alper et al., 1988) and other cells, and AE3 from brain and heart (Kopito et al., 1989; Kudrycki, Newman & Shull, 1990). The activity of band 3 is not strongly pH dependent (Gunn et al., 1973; Funder & Wieth, 1976), whereas Lee, Gunn and Kopito (1991) recently found that the gene products of AE2 and AE3 transfected into the human cell line 293 were increased in activity when the pH rose above 7.6. This value is above the physiological range in most cells and, in fact, the resting pH_i in transfected and nontransfected 293 cells was estimated to be 6.9–7.0. The authors point out that the cells showed signs of stress after transfection, which could be the reason for the high value required.

The anion transport protein conducting Na^+-*in*dependent Cl^-/HCO_3^- antiport in Vero cells has not been identified. Our preliminary studies show the existence in Vero cells of a transcript sharing sequence homology with AE1, AE2 and AE3. AE2 gave the best signal. Also, the comparatively high concentration of DIDS required to inhibit the AE2 antiporter (Lee et al., 1991) suggests that the Vero antiporter is most closely related to AE2.

All the effects of heat shock on the pH-regula-

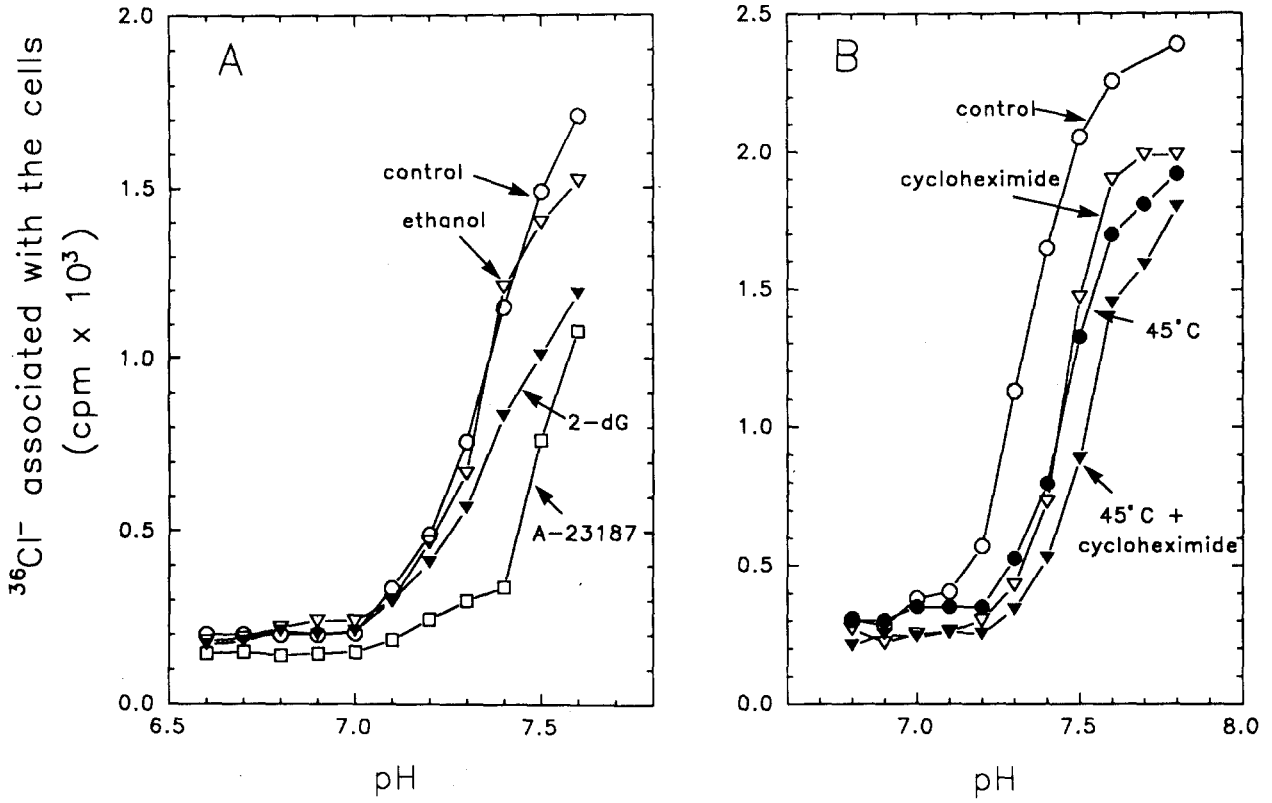

Fig. 7. Effect of HSP inducers and cycloheximide on Na^+-*in*dependent $^{36}Cl/Cl^-$ exchange. (*A*) Cells were incubated in HEPES medium, pH 7.4, containing 0.5% ethanol, 10 mM 2-deoxyglucose (2-dG), 10 μM A-23187 or no addition (control) for 2.5 hr. In (*B*) the cells were incubated for 15 min in HEPES medium and 36 μM cycloheximide was added when indicated and the incubation was continued for 10 min more. The incubation was then continued for 35 min either at 37 or at 45°C. In both *A* and *B* the cells were subsequently transferred to KCl buffer with the indicated pH, 2 μM nigericin and the same additions as those present during the preincubation. The cells were incubated at 37°C for 15 min. $^{36}Cl^-$ uptake was finally measured in mannitol buffer for 2.5 min as described in Materials and Methods.

tory mechanisms in Vero cells described here would tend to alkalinize the cytosol. When pH_i was measured in the absence of HCO_3^-, pH_i was substantially lower after heat shock. The reason for this acidification is not clear. While several authors have manipulated pH_i to measure the effect on thermal sensitivity, there are few studies on the effect of heat shock on pH_i. Cook and Fox (1988*abc*) found that in the absence of HCO_3^-, pH_i in CHO cells was increased by 0.05–0.1 pH units, 1 hr after heat shock was given at neutral pH_o, whereas Chu and Dewey found that pH_i in CHO cells was unaffected by hyperthermia (Chu & Dewey, 1987, 1988; Wang et al., 1990). Other authors demonstrated acidification of 0.15–0.35 pH units in mammalian cells upon heat shock (Aickin & Thomas, 1977; Yi et al., 1983). Recent reports have shown that in two different cell lines pH_i was reduced after exposure to heat, both in the absence and presence of HCO_3^- (Kiang, McKinney & Gallin, 1990; Lyons, Kim & Song, 1992). Our data indicate that in Vero cells, at least in the absence of HCO_3^-, heat shock leads to acidification of the cytosol.

There is an apparent inconsistency between the results in Figs. 1 and 2. Thus, the effect of heat shock on $^{36}Cl^-$ uptake was greater, especially at low pH_o values when pH_i was not clamped (Fig. 1) than when it was clamped by nigericin and isotonic KCl (Fig. 2). This is probably due to the acidification of the cytosol obtained after heat shock in the absence of HCO_3^-. In Fig. 1 the inhibitory effect of the reduced pH_i was presumably superimposed on the direct inhibition of the antiport by the heat shock.

Our data indicate that the effect of heat shock on ion transport does not appear as a result of HSP synthesis. First, the minimal time required to obtain inhibition (~5 min) is shorter than that expected if HSP were to be synthesized first. Furthermore, several agents known to induce HSP synthesis did not increase the critical pH for activation of the Na^+-*in*dependent Cl^-/HCO_3^- exchanger. An exception was the calcium ionophore A-23187 that shifted

the pH curve to higher pH values in a similar manner as heat shock. The effect occurred rapidly, and it was evident already after 10 min preincubation with the ionophore. The effects of heat shock and A-23187 were additive at optimal concentrations of the ionophore, indicating that they were due to actions on independent regulatory mechanisms.

^{35}S-methionine labeling of the cells revealed that protein synthesis was strongly reduced in the heat-shocked cells and no induction of new proteins could be detected after 15 min at 45°C. HSP synthesis was induced, however, after 3 hr at 41°C and after arsenite treatment (*unpublished data*). This indicates that induction of heat shock proteins cannot account for the rapid effect observed on the antiports.

Finally, the conclusion that HSP synthesis is not required for the effect is supported by the finding that inhibition of protein synthesis by cycloheximide and puromycin did not counteract the effect of heat shock on anion antiport. On the contrary, these agents, as such, were able to elicit the effect. Possibly, a protein with a short turnover is required to maintain normal regulation of the antiport. Both heat shock and treatment with protein synthesis inhibitors could result in rapid depletion of this protein. It should be noted that in Vero cells, the total protein synthesis after heat shock was reduced by approximately 45%.

We have found earlier that short-term treatment with the protein kinase C activator TPA has an effect on the Na^+-*in*dependent Cl^-/HCO_3^- antiport opposite to that of heat shock (Ludt et al., 1991). Both the effect of protein synthesis inhibitors and the effect of high temperature on ion transport could be counteracted by short-term treatment of the cells with TPA (*our unpublished results*). It is therefore possible that phosphorylation of the antiporter or of an associated protein is regulating its pH sensitivity. A short-lived kinase, whose synthesis is suppressed by heat shock could be required to maintain normal pH_i sensitivity of the antiporter.

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