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Effects of Hyperthermia on Intracellular Chloride

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Abstract. Hyperthermia induces transient changes in $[Na^+]_i$ and $[K^+]_i$ in mammalian cells. Since Cl^- flux is coupled with Na^+ and K^+ in several processes, including cell volume control, we have measured the effects of heat on $[Cl^-]_i$ using the chloride indicator, MQAE, with flow cytometry. The mean basal level of $[Cl^-]_i$ in Chinese hamster ovary cells was 12 mM. Cells heated at 42.0° or 45.0°C for 30 min had about a 2.5-fold increase in $[Cl^-]_i$ above unheated control values when measured immediately after heating. There was about a 3-fold decrease in $[Na^+]_i$ under the same conditions, as measured by Sodium Green. The magnitude of the increase in $[Cl^-]_i$ depended upon time and temperature. The $[Cl^-]_i$ recovered in a time-dependent fashion to control values by 30 min after heating. When cells were heated at 45.0°C for 30 min in the presence of 1.5 mM furosemide, the heat-induced $[Cl^-]_i$ increase was completely blocked. Since furosemide inhibits the $Na^+/K^+/2Cl^-$ cotransporter, Cl^- channels, and even Cl^-HCO_3 exchange, these ion transporters may be involved in the heat-induced increase in $[Cl^-]_i$.

Key words: Hyperthermia — MQAE — Intracellular chloride — Intracellular sodium — Furosemide — Flow cytometry

Introduction

Hyperthermia has long been thought to cause at least some of its effects by alterations at the level of the plasma membrane [14,15]. Previous reports have shown that hyperthermia causes alterations in intracellular free sodium ($[Na^+]_i$) [25,28], potassium [31,35], membrane fluidity [10,11] and membrane potential [5,28]. Our recent studies also show similar heat-induced changes in $[Na^+]_i$ and membrane potential [2], although these changes are probably not related to either hyperthermic cell killing or thermotolerance. Thus, the physiological role of thermally induced alterations in $[Na^+]_i$ and membrane potential has not yet been determined.

Since Na^+, K^+, and Cl^- fluxes are integrally involved in cell volume regulation via osmotic control [1,6,19,20,26,32], we hypothesized that the heat-induced changes in Na^+ and membrane potential may also be related to cellular osmotic control. To test this hypothesis, we measured the effects of hyperthermia on intracellular chloride ($[Cl^-]_i$). Heat-induced changes in $[Na^+]_i$ and $[K^+]_i$ are similar to the events which occur in RVI (regulatory volume increase). During RVI, a net uptake of KCl is observed which involves the Na^+/K^+ ATPase pump [13,20–32] and the $Na^+/K^+/2Cl^-$ cotransport system, which can be inhibited by furosemide [8,13,18,21,27,30].

In this study, the chloride indicator MQAE was used with flow cytometry to measure the effects of hyperthermia on the $[Cl^-]_i$ of Chinese hamster ovary cells. The effect of furosemide on heat-induced $[Cl^-]_i$ changes was also evaluated.

Materials and Methods

Cell Culture

A mutant Chinese hamster ovary cell line (IS1) [36] was used in this study. These cell have the same heat response as CHO cells, but retain a variety of physiological stains much better than CHO cells. Cells were routinely grown in Ham's F12 medium supplemented with 10% fetal bovine serum at 37°C in a 5% CO_2 incubator. Only cells in exponential growth were used for experiments.

Cell Loading with MQAE

Cells were trypsinized and counted using an electronic cell counter (Particle Data, Elmhurst, IL). One million cells were centrifuged, then

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resuspended in 1 ml hypotonic loading buffer containing 5 mM MQAE (Molecular Probes, Eugene, OR) and incubated at 37°C for 3 min [33]. Cells were then centrifuged and resuspended in either various calibration buffers (*described below*) or Ham's F12 medium with 2 mM sodium bicarbonate and 10 mM HEPES, pH 7.3, without phenol red or serum. All measurements were made at least 15 min after loading to allow cells to recover from hypotonic shock. The hypotonic loading buffer (150 mOsm) contained 65 mM NaCl, 1.2 mM $CaCl_2$, 0.6 mM $MgCl_2$, 10 mM HEPES, and 5 mM KCl, pH 7.3.

Heat and Chemical Treatments

Cells were loaded with MQAE, as described above, before heat and chemical treatments. Samples were heated in suspension using a temperature-regulated water bath (±0.05°C), and analyzed immediately unless otherwise specified. Control samples were kept at room temperature following loading throughout each experiment. Furosemide (Sigma, St. Louis, MO) was dissolved in Ham's F12 (serum-free) at a concentration of 1.5 mM. Cells were resuspended in medium containing 1.5 mM furosemide immediately prior to heating. All samples were run on the flow cytometer at room temperature within 90 min after loading.

Flow Cytometry

The cells were analyzed using an EPICS V cell sorter (Coulter, Miami, FL) interfaced to a Cicero data acquisition and display system (Cytomation, Ft. Collins, CO). MQAE was excited with 100 mW at 351–364 nm using an argon ion laser. Sodium Green was excited with 500 mW at 488 nm. Fluorescence at wavelengths above 408 nm (MQAE) or 515 nm (Sodium Green) was measured on a photomultiplier tube, and forward angle light scatter (FALS), which is related to cell size, was measured as a second parameter. The ratio of fluorescence to FALS was calculated for each cell using the Cicero software and collected as a histogram [3]. Mean values of this ratio histogram were used for calibration to obtain chloride concentration. Thirty thousand cells were measured from each sample.

Calibration of Intracellular Chloride

Calibration of the ratio channel number in terms of intracellular chloride was accomplished with the K^+-H^+ ionophore nigericin (Sigma, St. Louis, MO) and the Cl^--OH^- ionophore tributyltin chloride (Aldrich, Milwaukee, WI) [33]. High-K^+ calibration solutions were made from appropriate mixtures of high Cl^- and high gluconate solutions. The high Cl^- solution contained (in mM): 20 NaCl, 1.2 $CaCl_2$, 0.6 $MgCl_2$, 10 HEPES, and 120 KCl. The high gluconate solution was the same, except NaCl and KCl were replaced by sodium gluconate and potassium gluconate, respectively. Solutions were adjusted to pH 7.3 using NaOH. Calibration was performed by adding 5 μM nigericin and 10 μM tributyltin chloride to cells in 1 ml calibration buffers of various $[Cl^-]$. Nigericin stock solutions were 1 mM in 100% ethanol and tributyltin chloride stock solutions were 1.84 mM in DMSO. Five μl nigericin stock solution and 5.4 μl tributyltin chloride stock solution were added to stained samples 5–25 min before measurement. Ionophore incubation times depended upon the concentration of chloride in the calibration buffer [33].

Observed values of the ratio channel number F (fluorescence/FALS) at 5, 25, and 50 mM $[Cl^-]$ were used with the Stern-Volmer equation [33,34] in order to solve for unknown $[Cl^-]_i$. The Stern Volmer equation is:

$$(F_0/F) - 1 = K_{sv}[Cl^-] \quad (1)$$

where F_0 is the ratio channel number at $[Cl^-]_i = 0$ and K_{sv} is the Stern-Volmer constant. The ratio values obtained using three calibration samples of different $[Cl^-]$ were used to derive a fit to the Stern-Volmer equation with linear regression analysis.

Cell Loading and Calibration with Sodium Green

$[Na^+]_i$ measurements using Sodium Green with flow cytometry have been previously described [3]. Briefly, 1×10^6 cells were centrifuged, then resuspended in 1 ml loading buffer (Ham's F12, serum-free) containing 10 μM Sodium Green tetraacetate and 0.05% Pluronic (Molecular Probes, Eugene, OR) and incubated at 37°C for 1 hr. Following dye loading, cells were centrifuged and resuspended in either fresh medium (for heating experiments) or calibration buffers. Calibration was achieved using the ionophore, gramicidin D (Sigma, St. Louis, MO) and appropriate mixtures of high Na^+ and high K^+ solutions. Cells were incubated in 5 μM gramicidin D in calibration buffers for 30 min at room temperature before measurement.

Results

Flow cytometry histograms from a typical calibration of $[Cl^-]_i$ in CHO IS1 cells using MQAE are shown in Fig. 1. The fluorescence/FALS ratio method described previously for Sodium Green [3] was also used for the $[Cl^-]_i$ measurements. Cell size, as measured by FALS, was also measured 15 min after hypotonic dye loading (*data not shown*). Cells which were loaded with MQAE had the same FALS distribution as control cells without MQAE, and appeared normal under microscopic observation. Thus, the loading procedure did not have any apparent effect on cell size.

A typical Stern-Volmer calibration plot, derived from the mean values of ratio histograms such as those represented in Fig. 1, is shown in Fig. 2. F is the ratio channel number obtained by taking the ratio of fluorescence/FALS for each cell measured. The value of F_o was obtained by extrapolating the linear portion of the ratio channel number *vs.* $[Cl^-]_i$ calibration curve to $[Cl^-] = 0$, using linear regression analysis. This was done since it was not possible to make a calibration solution with the proper ingredients which has zero chloride; the high gluconate solution used was actually 3.6 mM $[Cl^-]$. Each experiment performed was calibrated using 5, 25, and 50 mM $[Cl^-]$ buffers. Using this calibration procedure, the mean $[Cl^-]_i$ in CHO IS1 cells was 12.4 mM with SEM = 1.21, from 3 experiments on different days.

The temperature dependence of $[Cl^-]_i$ and $[Na^+]_i$ was then evaluated in CHO IS1 cells (Fig. 3). The shapes of these curves are complementary, which implies that the same phenomenon may underlie both of these heat-induced effects. There was a remarkable symmetry between these two responses, especially after 41°C at which there is an inflection point in both curves. At this point, $[Na^+]_i$ increases, and $[Cl^-]_i$ decreases by a small but significant and reproducible amount compared to the values at lower temperatures.

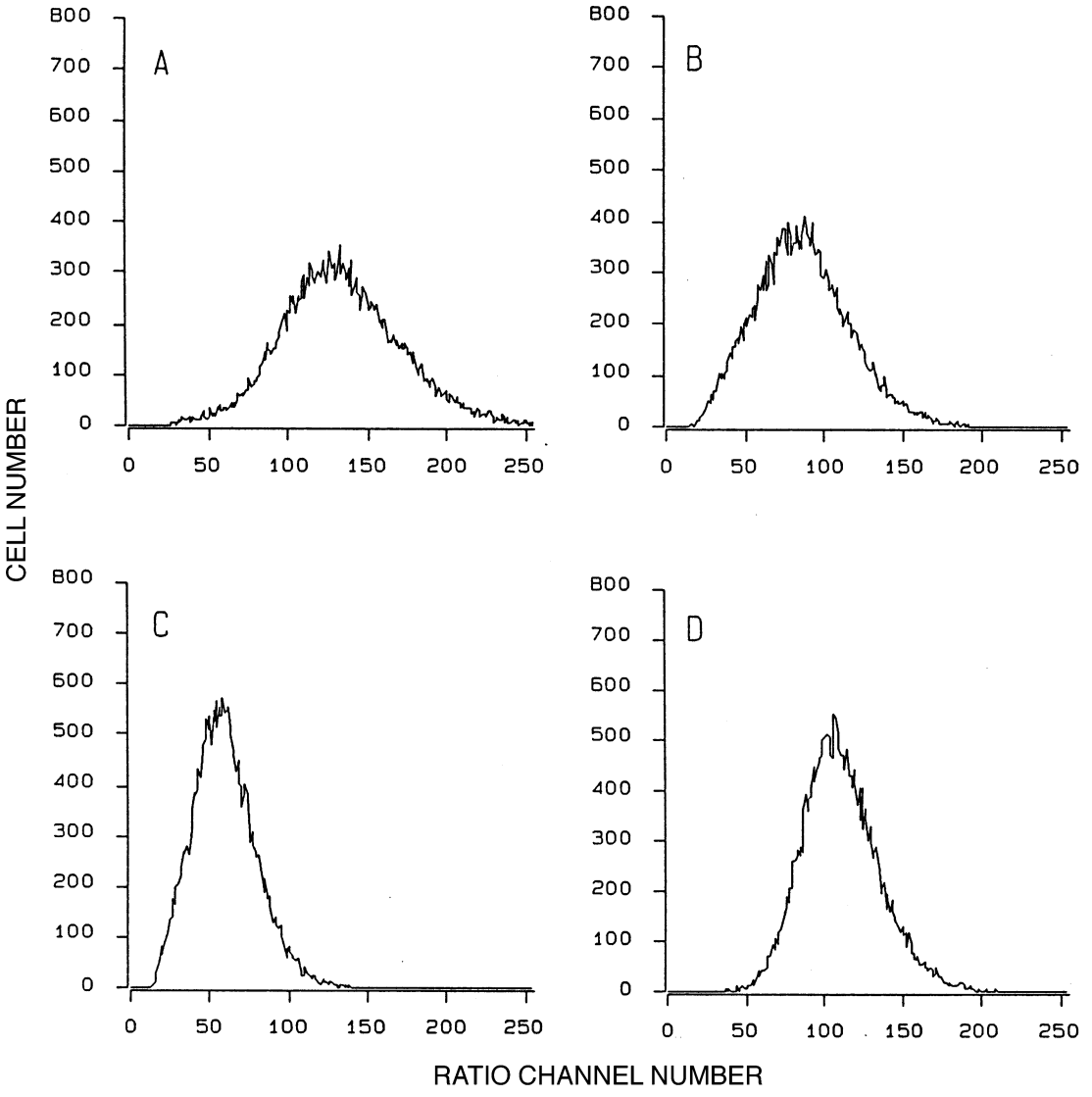

Fig. 1. Flow cytometry histograms of ratio channel number *vs.* $[Cl^-]_i$. Ratio channel number is proportional to the ratio of MQAE fluorescence to FALS. Cell suspensions were loaded with MQAE, centrifuged, and incubated with nigericin and tributyltin chloride at various chloride concentrations. (*A*) 5 mM $[Cl^-]$; (*B*) 25 mM $[Cl^-]$; (*C*) 50 mM $[Cl^-]$; (*D*) control (without nigericin or tributyltin chloride, in medium).

The magnitude of the change in $[Cl^-]_i$ was dependent upon heating time at both 42.0° and 45.0°C (Fig. 4). The changes observed in $[Cl^-]_i$ were similar at both 42.0° and 45.0°C, although there was a slightly larger effect at 42.0°C. The difference in $[Cl^-]_i$ induced by heating at 42.0° or 45.0°C is analogous to the difference in $[Na^+]_i$ between these two temperatures [2]. However, there was a lag time for this effect at both temperatures for $[Cl^-]_i$, in contrast to the rapid response observed in $[Na^+]_i$. Cell size did not change significantly after either heat treatment (*data not shown*).

To determine whether the changes in $[Cl^-]_i$ were transient, we measured $[Cl^-]_i$ at various times after heating. $[Cl^-]_i$ recovered to control values by about 20 min after heating at 45.0°C for 30 min (Fig. 5). The kinetics of this recovery were similar to the recovery in $[Na^+]_i$ and membrane potential after heating [2], though again there was a delay in the response of $[Cl^-]_i$, which was not seen for $[Na^+]_i$.

Furosemide, an inhibitor of the $Na^+/K^+/2Cl^-$ cotransporter [13,18], was added to some samples prior to heating, and cells were analyzed immediately after heat treatment (Table). When 1.5 mM furosemide was present during heating at 45.0°C for 30 min, the 2-fold increase in $[Cl^-]_i$ was blocked completely. There was also a small decrease in the $[Cl^-]_i$ of unheated control cells, which is expected [16,23]. However, the magnitude of this effect in control cells did not account for the large inhibition of the heat-

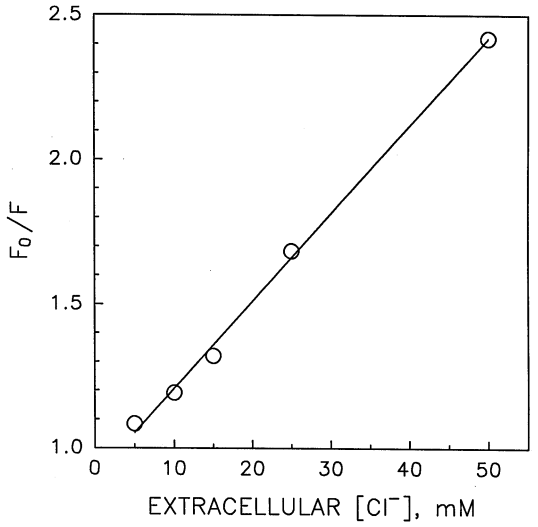

Fig. 2. Stern-Volmer plot of MQAE calibration using flow cytometry. CHO IS1 cells were loaded with MQAE, centrifuged, and resuspended in buffers of various [Cl^-]. The ionophores nigericin and tributyltin chloride were used such that $[Cl^-]_i = [Cl^-]_o$. F is the mean ratio channel of fluorescence/FALS for various values of $[Cl^-]_i$ and F_o is the extrapolated ratio of fluorescence/FALS at $[Cl^-]_i = 0$.

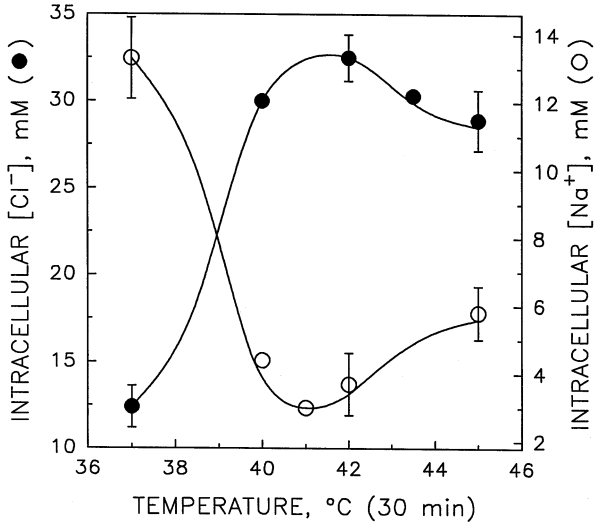

Fig. 3. Temperature dependence of $[Cl^-]_i$ and $[Na^+]_i$. CHO IS1 cells were loaded with MQAE or Sodium Green, heated for 30 min at various temperatures, and analyzed immediately. Unheated controls were kept at room temperature. Error bars represent the SEM values from three independent measurements. (●), $[Cl^-]_i$ using MQAE; (○), $[Na^+]_i$ using Sodium Green.

induced $[Cl^-]_i$ increase. The $[Na^+]_i$ was reduced even further in the presence of furosemide, however.

Discussion

The effects of hyperthermia on $[Cl^-]_i$ were measured in this study using the chloride indicator, MQAE, with flow

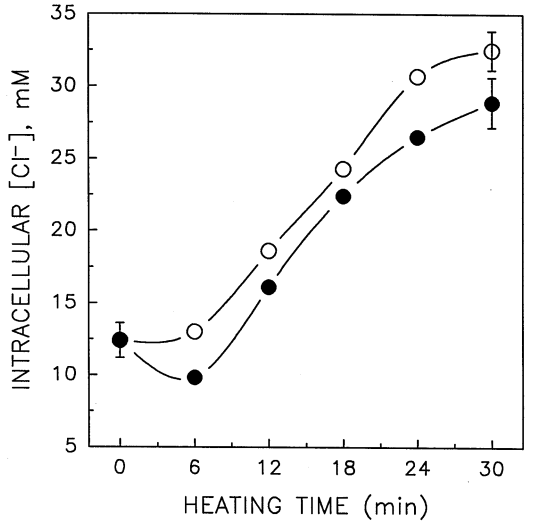

Fig. 4. Dependence of the $[Cl^-]_i$ upon heating time. Cells were loaded with MQAE, heated at 42.0° or 45.0°C for various times, and analyzed immediately. Error bars represent SEM values from three experiments. (○), 42.0°C; (●), 45.0°C.

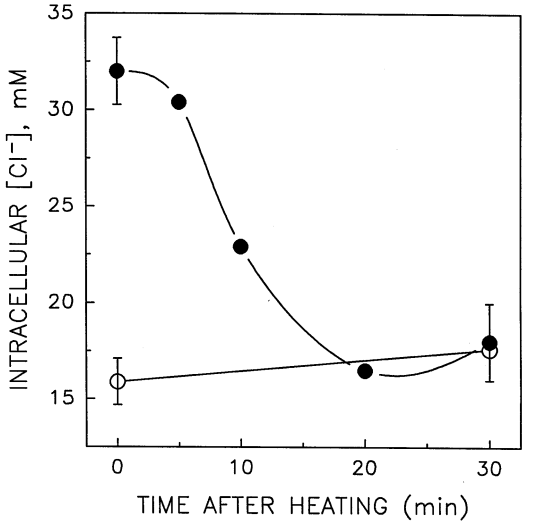

Fig. 5. Time-dependent recovery of $[Cl^-]_i$ to control values after heating. Cells were loaded with MQAE, heated at 45.0°C for 30 min, and analyzed at various times after heating. Unheated control cells were kept at room temperature. Error bars represent SEM values from three experiments. (●), heated; (○), unheated control.

cytometry. Cells heated at 42.0° or 45.0°C for 30 min had about a 2-fold increase in $[Cl^-]_i$ above unheated control values when measured immediately after heating. The heat-induced increase was dependent upon time of heating and temperature. Intracellular chloride levels returned to control values by 20 min after heating at 45.0°C for 30 min. Furosemide (an inhibitor of the $Na^+/K^+/2Cl^-$ cotransporter), when present during heating, blocked the heat-induced increase in $[Cl^-]_i$.

Table. Effects of 1.5 mM furosemide on the heat-induced changes in intracellular sodium and chloride. CHO IS1 cells were heated for 30 min at 45.0°C, then analyzed immediately. $[Na^+]_i$ was measured with Sodium Green and $[Cl^-]_i$ was measured with MQAE.

Sample	$[Cl^-]_i$ mM	$[Na^+]_i$ mM
Control (Unheated)	12.7	22.6
Control + furosemide	7.0	18.7
Heated	26.0	6.0
Heated + furosemide	9.5	2.6

Intracellular chloride measurements have been made using radioactive ion analogues [17], analysis of cell supernatant using chloride selective electrodes [22], and ion-sensitive microelectrodes [4,7]. MQAE was chosen for this study based upon its sensitivity to chloride and ease of loading into cells [33,34]. MQAE has also been used recently by other investigators [9,12,16,23]. MQAE measurements are obtained using a single excitation wavelength, and are not inherently normalized for dye content within cells. However, we have used a fluorescence/FALS ratio technique described previously [3] to normalize for cell size variations. This was necessary since there are no chloride indicators available which can be used with a fluorescence ratio technique. As with all techniques such as this, there is the possibility of artifacts caused by dye leakage. In the case of MQAE, dye leakage would give a smaller fluorescence signal, indicating a higher $[Cl^-]_i$. This probably explains the slight increase in $[Cl^-]_i$ in unheated control cells shown in Fig. 5. This could not possibly explain the heat-induced increase in $[Cl^-]_i$ however, since it is reduced back to unheated control levels by 30 min after heating. If dye were leaking out, the apparent $[Cl^-]_i$ would continue to increase.

The typical range of $[Cl^-]_i$ values for nucleated mammalian cells is 5–15 mM [1]; our measured mean value of 12 mM is well within this range. There has been only one previous report describing the effects of hyperthermia on $[Cl^-]_i$ [4]; this group used microelectrodes to measure a mean baseline $[Cl^-]_i$ of 18 mM in murine neuroblastoma cells. No significant effects on $[Cl^-]_i$ due to a 45.5°C, 30-min heat treatment were found in their study. However, they did not measure the effects of this heat treatment on $[Cl^-]_i$ immediately after heating, but showed the effects as 0–4 hr post heat (the time taken to make microelectrode measurements on 50 cells). We showed that the heat-induced changes in $[Cl^-]_i$ recovered to baseline values within 20 min, so they would have missed these changes. Since flow cytometry can be used to make measurements on tens of thousands of cells within minutes of heating, our results are also more accurate.

There is a remarkable inverse correlation between the temperature dependence of $[Na^+]_i$ and that of $[Cl^-]_i$ in cells heated for 30 min. The complementary nature of these two curves implies that the same process may be involved in both of these effects. However, there is a difference in the kinetics of the changes and in their recovery after heating. There was no change in $[Cl^-]_i$ after heating 6 min at 45.0°C, yet $[Na^+]_i$ had decreased 50% by the same time of heating [2]. By 5 min after heating, $[Na^+]_i$ recovered to 50% of preheated values [2], but $[Cl^-]_i$ did not recover at all (Fig. 5). This difference in kinetics may mean that different transporters and/or pumps are involved in transporting Na^+ and Cl^-.

The fact that furosemide blocks the heat-induced increase in $[Cl^-]_i$ and leads to an even greater heat-induced decrease in $[Na^+]_i$ (Table) implies that heat affects the $Na^+/K^+/2Cl^-$ cotransporter, since furosemide inhibits this cotransport system [8,13,18,21,27,30]. Ouabain, an inhibitor of the Na^+/K^+ATPase, blocks the decrease in $[Na^+]_i$ after heating [2]. Thus, both of these pumps are likely involved in the heat-induced changes in Na^+ and Cl^-. When the $Na^+/K^+/2Cl^-$ cotransporter is blocked with furosemide, Na^+ would not be pumped into the cell with this cotransporter and the $[Na^+]_i$ would fall even lower, which is precisely what was observed (Table). Furosemide could also cause other effects, however, such as inhibiting Cl^- channels or Cl^-/HCO_3^- exchange.

The changes in sodium and chloride reported here in response to hyperthermia, along with observations we have made on membrane potential [2] (and thus potassium), are similar to changes in ions as a result of regulatory volume increase (RVI) in response to osmotic shock [18,24]. The RVI response is dependent upon the $Na^+/K^+/2Cl^-$ cotransporter and the Na^+/K^+-ATPase, which can be inhibited by furosemide and ouabain, respectively. We have shown here that furosemide inhibits the changes in chloride and previously shown that ouabain inhibits the changes in sodium and potassium [2]. It is possible that the cells are responding to heat as if they were osmotically shocked, and try to adjust by ion flow. This hypothesis is unproven at the present time, and further work would be necessary to validate it.

Minton *et al.* [29] has proposed a model to account for large increases in transporter-mediated ion flux across cell membranes based on macromolecular crowding. The model is dependent upon a balance between phosphorylated and nonphosphorylated states of transporters that regulate their activity. Cell swelling, or other factors that affect macromolecular crowding, results in the inhibition of kinase relative to phosphatase activity, and thus modulate the activity of ion flux. Hyperthermia might be causing macromolecular crowding effects that could lead to the ion changes observed, according to this model. Again, this is speculation, and further experiments would be necessary to confirm or reject the hypothesis.

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