Temperature dependence of Na+–H+ exchange, Na+–HCO3− co-transport, intracellular buffering and intracellular pH in guinea-pig ventricular myocytes

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Almost all aspects of cardiac function are sensitive to modest changes of temperature. We have examined the thermal sensitivity of intracellular pH regulation in the heart. To do this we determined the temperature sensitivity of pH_i, intracellular buffering capacity, and the activity of sarcolemmal acid-extrusion proteins, Na^+–H^+ exchange (NHE) and Na^+–HCO_3^- co-transport (NBC) in guinea-pig isolated ventricular myocytes. pH_i was recorded fluorimetrically with acetoxymethyl (AM)-loaded carboxy-SNARF-1 at either 27 or 37 °C. At 27 °C, intrinsic (non-CO_2-dependent) buffering power (β_i) was ~60 % of that at 37 °C. Acid-extrusion (J^e) through NHE was ~50 % slower than at 37 °C, consistent with a Q_{10} of ~2. In 5 % CO_2/HCO_3^--buffered conditions, in the presence of 30 μM cariporide to inhibit NHE, acid extrusion via NBC was also slowed at 27 °C, suggestive of a comparable Q_{10}. Resting pH_i at 27 °C was similar in Hepes- or 5 % CO_2/HCO_3^--buffered superfusates but, in both cases, was ~0.1 pH units lower at 37 °C. The higher the starting pH_i, the larger was the thermally induced fall of pH_i, consistent with a mathematical model where intrinsic buffers with a low principal pK_a (e.g. close to 6.0) are less temperature-sensitive than those with a higher pK_a. The high temperature sensitivity of pH_i regulation in mammalian cardiac cells has implications for experimental work conducted at room temperature. It also has implications for the ability of intracellular acidosis to generate intracellular Na^+ and Ca^{2+} overload, cardiac injury and arrhythmia in the heart.

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Temperature is an important modulator of intracellular protein activity. One category of protein is the group of membrane ion transporters that tightly regulates intracellular pH (pH_i). In mammalian cardiac cells, for example, this group includes the sarcolemmal, acid-equivalent extrusion proteins, Na^+–H^+ exchange (NHE) and Na^+–HCO_3^- co-transport (NBC) (Leem *et al.* 1999). Intracellular pH is also an important modulator of cell function, affecting contractile (Bountra & Vaughan-Jones, 1989) and electrical activity in the heart (Orchard & Cingolani, 1994; Ch'en *et al.* 1998). Some effects of temperature on cellular activity may therefore be mediated indirectly through an influence on intracellular pH regulation.

Hypothermia (4–10 °C) is commonly used during cardiac surgical procedures (Hearse *et al.* 1981). In addition, basic experimental work on cardiac tissue is frequently carried out at room temperature. Many of the intracellular ionic changes associated with reduced temperature have yet to be extensively investigated. This is particularly pertinent in the heart, given the intimate homeostatic link between intracellular pH, Ca^{2+}, contractility and electrical rhythm. Overactivity of sarcolemmal Na^+–H^+ exchange (Scholz *et al.* 1995; Xue *et al.* 1996; Hartmann & Decking, 1999; Xiao

& Allen, 2000) and possibly NBC (Schafer *et al.* 2000) has been implicated in cardiac injury and arrhythmogenesis during intracellular acid-overload, observed typically during myocardial ischaemia and reperfusion (Karmazyn, 1999). It is of interest that, under these conditions, hypothermia confers some cardioprotection (Shipolini *et al.* 1997). Knowledge of the temperature dependence of NHE and NBC may be valuable in understanding the protective mechanisms involved.

A recent study in rat cardiomyocytes indicates that NHE activity is slowed considerably by moderate hypothermia (Hoshino & Avkiran, 2001). That report utilised extracellular superfusates buffered with Hepes rather than a physiologically relevant CO_2/HCO_3^- system. We have therefore studied effects of temperature on acid extrusion transporters in guinea-pig ventricular myocytes exposed to either Hepes- or CO_2/HCO_3^--buffered superfusates. This enables us to assess more comprehensively the effects of temperature on both NHE and NBC. The rate at which these transporters mediate a change of pH_i depends, in part, on intracellular buffering power. Intrinsic (non-CO_2-dependent) buffering has been variously reported to increase (Do *et al.* 1996; Hoshino & Avkiran, 2000), decrease (Graber *et al.*

1992; van Dijk *et al.* 1997) or remain unchanged (Kiang *et al.* 1990) following a temperature reduction. We therefore investigated the temperature sensitivity of intracellular buffering. Finally, the thermal sensitivity of pH_i was examined, given that changes in buffering and sarcolemmal acid transport may ultimately affect its steady state value. The consequences of the temperature dependence of pH_i regulation in the heart, particularly in relation to its effects on contractility and arrhythmogenesis, are discussed.

Preliminary reports of some of this work have been published in abstract form (Ch'en *et al.* 2000, 2001).

METHODS

Isolation of guinea-pig ventricular myocytes

The composition of cell isolation solutions and the detailed procedure have been previously described (Lagadic-Gossmann *et al.* 1992). In brief, single ventricular myocytes were isolated from 350–450 g albino guinea-pigs (killed by cervical dislocation; procedure conformed with the UK Animals (Scientific Procedures) Act 1986) using enzymatic and mechanical dispersion (0.7 mg ml^{-1} collagenase, Boehringer Mannheim and 0.04 mg ml^{-1} protease, Sigma). The cells were suspended in Hepes-buffered Dulbecco's Modified Eagle's Medium and left at room temperature (18–22 °C) until use. Only rod-shaped myocytes showing Ca^{2+} tolerance were used in this work.

Measurement of intracellular pH

SNARF loading and calibration. The intracellular pH of isolated ventricular myocytes was measured using the dual emission fluorophore carboxy-SNARF-1, loaded as the acetoxymethyl (AM) ester. The full procedure for dye loading, measuring pH_i, and calibrating dye signals has been described previously (e.g. Buckler & Vaughan-Jones, 1990; Sun *et al.* 1996). Briefly, isolated myocytes were loaded for 8 min at room temperature with 10 μM carboxy-SNARF-1 AM ester. SNARF fluorescence from individual cells was excited at 540 ± 12 nm and measured simultaneously at 590 ± 5 and 640 ± 5 nm with an inverted microscope converted for epifluorescence (Nikon Diaphot). Signals were digitised at 0.5 kHz (CED 1401) and the emission ratio calculated and converted to a pH_i value using the pH ratiometric fluorescence equation (Buckler & Vaughan-Jones, 1990; Sun *et al.* 1996). The pH calibration data for this equation were obtained *in situ* from individual cells using the nigericin (10 μM) technique (see below for calibration solutions) and averaged for more than 10 cells from at least three animals. The data were acquired routinely every 2 months for the terms R_{max} (maximum emission ratio at pH 5.5), R_{min} (minimum ratio at pH 9.5), and $F_{640,max/min}$ (9.5/5.5 pH fluorescence ratio measured at 640 nm), measured at both 27 and 37 °C. Typical values at 27 °C were 1.648, 0.266 and 1.698, respectively. These predict a $-\log$ dissociation constant (pK_a) of 7.43 for intracellular SNARF. Typical values at 37 °C were 1.623, 0.231 and 2.035, respectively. These predict a pK_a of 7.36 for intracellular SNARF. To reduce potential contamination of the cell superfusion system with nigericin (Richmond & Vaughan-Jones, 1997), nigericin calibration was not performed after every experiment; instead, the most recently acquired pH calibration data were used as default values.

Cleaning the superfusion system. The apparatus was thoroughly cleaned following a nigericin calibration. The superfusion lines were replaced and the superfusion chamber and switcher tap (see Richmond & Vaughan-Jones, 1997, for details of superfusion) were dismantled and soaked in ethanol for several hours. This was followed by a soak for at least 12 h in 20 % Decon 75 (Decon Laboratories Ltd, Sussex, UK), and then simmering in deionised water for several hours.

Solutions

Hepes-buffered Tyrode solution. Standard Hepes-buffered Tyrode solution contained (mM): NaCl 140, KCl 4.5, MgCl$_2$ 1, CaCl$_2$ 2, Hepes 20 and glucose 11. This was adjusted to pH 7.4 at 37 or 27 °C with 4 M NaOH.

CO_2/HCO_3^--buffered Tyrode solution. Standard CO_2/HCO_3^--buffered Tyrode solution pH 7.4 had the same composition as Hepes-buffered Tyrode solution but with [NaCl] reduced to 125 mM, and 22 mM NaHCO$_3$ substituted for Hepes. All CO_2/HCO_3^--buffered Tyrode solutions at pH 7.4 were equilibrated with 5 % CO_2 and 95 % air for at least 1 h prior to starting experiments.

Calibration solutions. Nigericin calibration solutions contained (mM): KCl 140, MgCl$_2$ 1, EGTA 0.5 and nigericin 0.01, buffered with one of the following organic buffers (Sigma, UK): 20 mM Mes (pH 5.5), 20 mM Pipes (pH 6.5), 20 mM Hepes (pH 7.5) or 20 mM Capso (pH 9.5), and were adjusted to the correct pH with 4 M NaOH at 37 or 27 °C.

Chemicals

When 15 mM ammonium chloride was used, it was added directly to solutions without osmotic compensation. Hoe 642 (cariporide) was kindly provided by Aventis (formerly Hoechst AG, Germany) and added as a solid to perfusates shortly before use. All other chemicals were from Sigma (UK) and Merck (UK).

Calculation of acid-equivalent fluxes

Acid-equivalent fluxes (J^e) were estimated as the product of the rate of pH_i recovery (dpH_i/dt) following an intracellular acid load (typically pre-pulsing with 15 mM NH$_4$Cl), and the total intracellular buffering power (β_{tot}).

The value of dpH_i/dt was determined by computer as the least squares regression line fitted to the initial change of calibrated pH_i sampled at 0.5 s intervals over a 0.5–1 min time period. The pH_i associated with the calculated dpH_i/dt was taken to be the mid-point pH_i of linear best fit. Note that comparisons of acid-equivalent fluxes in the present work were made at a common mid-point pH_i.

The term β_{tot} is the sum of intrinsic buffering power (β_i) plus buffering power due to CO_2/HCO_3^- (β_{CO_2}). β_i in guinea-pig ventricular myocytes at 37 °C has been estimated as:

$$\beta_i = \frac{[TA] \times \ln(10) \times 10^{(pH_i - pK_A)}}{(1 + 10^{(pH_i - pK_A)})^2} + \frac{[TB] \times \ln(10) \times 10^{(pH_i - pK_B)}}{(1 + 10^{(pH_i - pK_B)})^2},$$

(1)

where [TA] and [TB] are the total concentrations of buffer A and buffer B, respectively. The values for the parameters were calculated as: [TA] = 84.22 mM, [TB] = 29.38 mM, $pK_A = 6.03$ and $pK_B = 7.57$ (Leem *et al.* 1999), and have been used in the present work. The value for β_{CO_2} was estimated using the Henderson-Hasselbach equation as: $\beta_{CO_2} = 2.303[HCO_3^-]_i$ (Leem *et al.* 1999). This assumes the same pK_a for CO_2/HCO_3^- and the same solubility for CO_2 on either side of the sarcolemma.

Experimental determination of intracellular buffering power

In experiments where β_i (mM) was determined experimentally, intracellular acid loads of differing magnitude were induced by ammonium removal and the consequent change in pH$_i$ was used to compute intrinsic buffering power, β_i (Roos & Boron, 1981):

$$\beta_i = \Delta[H^+]_i/-\Delta pH_i.$$

Removing extracellular NH$_4^+$ results in the depletion of intracellular NH$_4^+$ that exits as NH$_3$, leaving behind H$_i^+$. Since [H$^+$]$_i$ can thus be equated with [NH$_4^+$]$_i$ at the moment of removal, the expression for β_i becomes:

$$\beta_i = \Delta[NH_4^+]_i/\Delta pH_i.$$

This can then be evaluated with [NH$_4^+$]$_i$ expressed as a rearrangement of the Henderson-Hasselbalch equation:

$$[NH_4^+]_i = C/(10^{(pH_o - pH_i)} + 1) \times 10^{(pH_o - pH_i)},$$

where C is the total extracellular concentration of the weak base and assuming the dissociation constant of NH$_4^+$ to be the same intracellularly and extracellularly at both 37 and 27 °C. Note that the method assumes a rapid equilibration of NH$_3$ across the sarcolemma and that during the acid loading phase, significant transmembrane transport of acid-equivalents does not occur.

By a similar method, prepulsing a cell with sodium acetate (salt of a weak acid) induces an intracellular alkalosis that may be used to estimate β_i. The concentration of intracellular base added is assumed to equal [Acetate]$_i$ at the end of the prepulse period:

$$[Acetate]_i = C/(10^{(pH_i - pH_o)} + 1) \times 10^{(pH_i - pH_o)},$$

where C is the total extracellular concentration of sodium acetate, assuming the pK_a of acetic acid remains unchanged within the cytoplasmic compartment.

Determination of bath temperature changes

To ensure that pH$_i$ measurements were made at the appropriate temperature, the time course of change in bath temperature on changing heater settings for the superfusate was measured. In standard Hepes-buffered solution, pH$_o$ 7.4, the heater temperature was step-changed from 27 to 37 °C and the bath temperature was monitored. Approximately 60 s was needed to raise the bath temperature to 37 °C (data not shown). All temperature-induced effects on pH$_i$ in the present work were therefore measured ~1 min after changing the heater temperature.

RESULTS

Thermal sensitivity of carboxy-SNARF-1 ratio

Given that we assess pH$_i$ regulation by monitoring intracellular SNARF fluorescence, we initially investigated the dye's temperature sensitivity. Figure 1A shows results from a typical series of calibration experiments of carboxy-SNARF-1 using the nigericin technique (Sun *et al.* 1996), performed at 27 and 37 °C (filled triangles and circles, respectively). The fluorescence ratios obtained were different at the two temperatures and, for a given pH$_i$, fell with an increase of temperature.

The dynamic range of the dye's fluorescence ratio increased with increasing temperature: R_{max}/R_{min} was 6.19 at 27 °C and 7.03 at 37 °C. These results are in good agreement with work

on carboxy-SNARF-1 in single mouse muscle fibres (Westerblad *et al.* 1997). The pK_a values of SNARF at the two temperatures were similar: 7.43 at 27 °C and 7.36 at 37 °C, and are consistent with previous estimates *in vitro* and intracellularly (Buckler & Vaughan-Jones, 1990; Blank *et al.* 1992; Westerblad *et al.* 1997).

Given the above temperature-dependent changes in the spectral properties of SNARF, care must be taken to use calibration data acquired at the relevant temperature. This is emphasised in Fig. 1B, which shows the direct effect of temperature on the intracellular SNARF ratio. The cell's pH$_i$ was clamped throughout the experiment at 7.0 by superfusing a high K^+_o solution containing 10 μM nigericin (see Methods). Lowering the temperature by 10 °C reversibly increased the SNARF ratio despite that fact that pH$_o$, and hence pH$_i$, would have been constant. In this case, the observed shift in the intracellular ratio was related directly
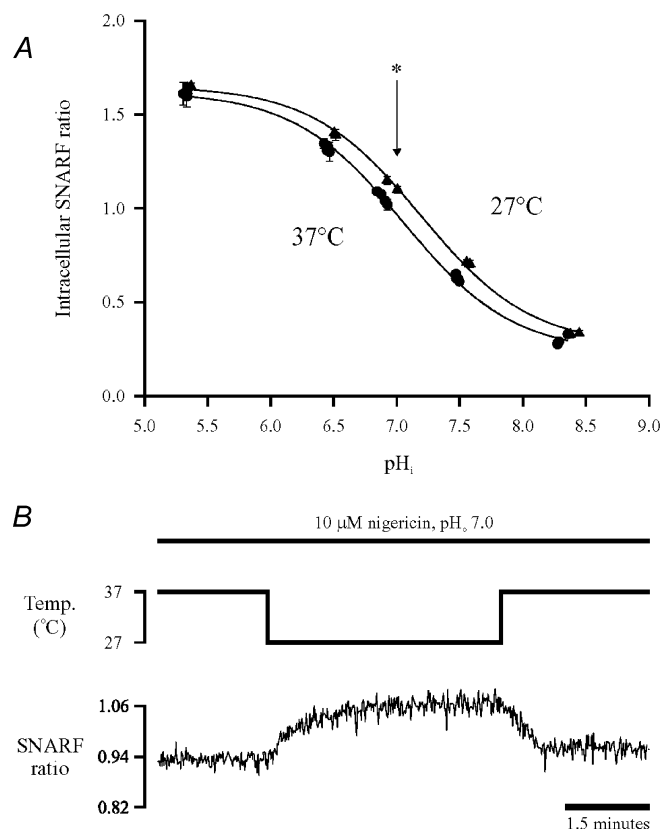

Figure 1. Temperature dependence of SNARF ratio

A, intracellular SNARF fluorescence ratio was calibrated by superfusing nigericin (10 μM) solutions of pH 5.5, 6.5, 7.0, 7.5 and 8.5 at 37 °C (●) and 27 °C (▲). The arrow indicates that at pH$_i$ values close to typical pH$_i$, decreasing temperature at a constant pH$_i$ causes an increase in SNARF ratio. *B,* temperature-dependent shifts of intracellular SNARF ratio that are independent of pH$_i$. Extracellular pH was kept constant at 7.0 in the presence of 10 μM nigericin while the temperature was decreased from 37 to 27 °C. Note that while pH$_i$ will be constant under these conditions, there was nevertheless a large increase in SNARF ratio.

to the temperature change and not to any change in pH_i. The two values of SNARF ratio obtained in Fig. 1*B* at 37 and 27 °C concurred with the calibration plots in Fig. 1*A*, as denoted by the asterisk. SNARF calibration parameters appropriate for a given temperature have therefore been used throughout the present work.

Thermal sensitivity of intrinsic buffering

To ensure the accurate evaluation of acid-equivalent efflux (J^e), the corresponding intrinsic buffering power (β_i) needs to be calculated at the same temperature. The value of β_i has recently been estimated over a wide pH_i range (6.2–7.7) at 37 °C (Leem *et al.* 1999; Zaniboni *et al.* 2003), but has not been established for 27 °C.

Figure 2 illustrates an experiment where intrinsic buffering power was estimated at both temperatures. A cell was superfused with Hepes-buffered Tyrode solution, i.e. nominally free of CO_2/HCO_3^-, in the presence of 30 μM cariporide (Hoe 642), a high affinity inhibitor of Na^+–H^+ exchange (Scholz *et al.* 1995), to prevent sarcolemmal acid efflux. In some experiments, Cl^- was also removed from the superfusate (gluconate-substituted) to inhibit acid-equivalent influx on sarcolemmal Cl^-–OH^- exchange (CHE), although this made no significant difference (data
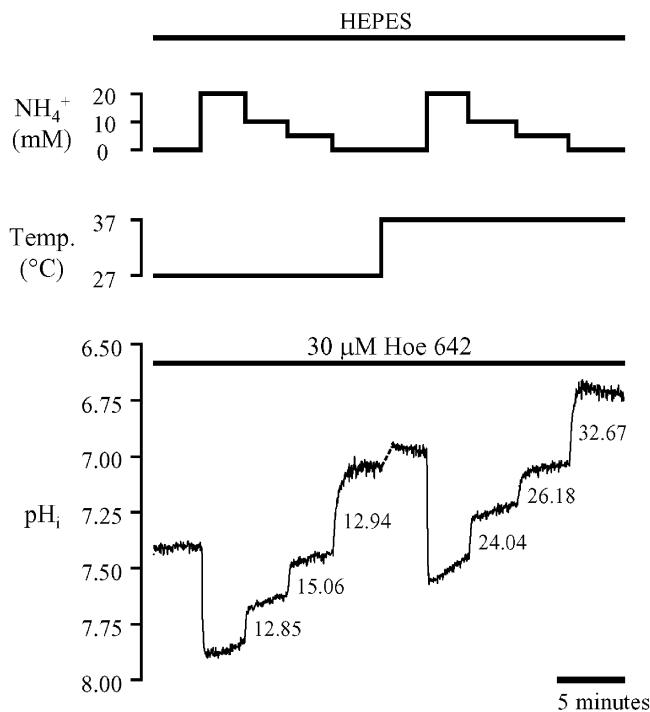

Figure 2. Ammonium prepulses at two temperatures
Stepwise intracellular acid loads were induced sequentially by superfusing with 20 mM NH_4Cl and then reducing extracellular ammonium concentration to 10, 5 and 0 mM, first at 27 °C and then again at 37 °C. All solutions contained 30 μM Hoe 642 to inhibit Na^+–H^+ exchange and were Hepes-buffered. (pH_o 7.4). Each stepwise fall of pH_i was used to calculate intracellular intrinsic buffering, β_i. The resulting value is listed for the mid-point of each step fall in pH_i.

not shown). The cell was acid-loaded by using the ammonium pre-pulse technique (see Methods), and the fall of pH_i was recorded at 27 and 37 °C. As illustrated in Fig. 2, incremental reduction of extracellular ammonium concentration during the pre-pulse period (40, 20, 10, 5 mM and zero) induced a step-wise fall of pH_i that was subsequently used to estimate β_i. In other experiments (not shown; see Methods), in order to measure β_i at higher values of pH_i, incremental reduction of extracellular acetate was used during the prepulse period (80, 40, 20 mM and zero) to induce a stepwise rise of pH_i. Significantly in all cases, the step-wise change in pH_i was larger at 27 °C, suggesting that at a common pH_i, β_i was reduced at the lower temperature.

Figure 3*A* shows estimates of β_i from experiments similar to that shown in Fig. 2 using ammonium withdrawal. These have been pooled with results using acetate withdrawal. Sample measurements of β_i performed at 37 °C are in good agreement with the relationship for intrinsic buffering power determined previously at this temperature (continuous grey line; Leem *et al.* 1999; calculated using eqn (1) in Methods of present paper; see also Zaniboni *et al.* 2003). For any given pH_i, the values of β_i measured at 27 °C are significantly lower. Intrinsic buffering at 27 °C, expressed as a percentage of that at 37 °C, is plotted in Fig. 3*B*. Over the pH_i range 7.6–6.5, buffering capacity is reduced during hypothermia by about 40 %.

Thermal sensitivity of Na^+–H^+ exchange

The effect of a 10 °C decrease on the activity of Na^+–H^+ exchange was examined by observing pH_i recovery following an intracellular acid load induced by 15 mM NH_4Cl, first at 37 °C and then at 27 °C. The recovery in Hepes-buffered Tyrode (nominally free of CO_2/HCO_3^-) is mediated by acid-extrusion through NHE (Lagadic-Gossmann *et al.* 1992). A representative experiment is shown in Fig. 4*A*. This demonstrates a significant slowing at 27 °C, indicating a reduction in NHE activity.

Figure 4*B* pools data obtained from several experiments similar to that shown in Fig. 4*A*. Using β_i values appropriate for each temperature and pH_i (obtained from the curves plotted in Fig. 3*A*), NHE-mediated acid extrusion ($dpH_i/dt \times \beta_i$) was plotted *versus* pH_i at 27 °C (filled circles) and at 37 °C (open circles). Inspection of the flux data in Fig. 4*B* indicates an approximate halving of NHE activity on reducing temperature. Calculating the quotient of the two activities at the two temperatures gives the apparent Q_{10} ((acid efflux at 37 °C)/(acid efflux at 27 °C)). This is plotted in Fig. 4*C* for the pH_i range 6.6–7.1 and is ~2.0. Over most of the pH_i range, the Q_{10} appeared to be relatively constant.

Assuming that activation energy, E_a, for NHE is independent of temperature, the integrated Arrhenius equation is: $\ln(k_{T_1}/k_{T_2}) = -E_a/R(1/T_1 - 1/T_2)$, where R is the gas constant

and k_{T_1} and k_{T_2} are the rate constants at temperatures T_1 and T_2, respectively. With the calculated rates of acid efflux at 27 and 37 °C, this gives an activation energy of 55.1 kJ mol^{-1}.

Thermal sensitivity of Na$^+$–HCO$_3^-$ co-transport

Figure 5*A* illustrates an experiment similar to that shown in Fig. 4*A*, but performed in 5 % CO_2/HCO_3^- rather than Hepes buffer. Under these conditions, the recovery from an acid load induced by 15 mM NH$_4$Cl removal is mediated by both NHE and NBC (Lagadic-Gossmann *et al.* 1992; Dart & Vaughan-Jones, 1992). To examine NBC activity in isolation, NHE activity was eliminated by adding 30 μM cariporide to the superfusate. The pH$_i$ recovery rate following ammonium removal was significantly slower at 27 °C, indicating a reduction in NBC activity.

Figure 5*B* is a plot of data pooled from several experiments similar to those shown in Fig. 5*A*, including experiments where the order of the imposed temperatures was reversed, i.e. first 37 °C then 27 °C; there was no significant difference between the two protocols (data not shown). Acid-equivalent efflux has been plotted as a function of pH$_i$ at both 27 °C (filled circles) and 37 °C (open circles), and as with NHE activity, there was roughly a halving of activity on reducing temperature by 10 °C. Figure 4*C* shows the apparent Q_{10} ((acid-equivalent efflux at 37 °C)/(acid-equivalent efflux at 27 °C)) for NBC over a

range of pH$_i$ values. The apparent Q_{10} was, again, largely constant over the range and had a value of ~2.4. Using the integrated Arrhenius equation above, these data give an activation energy for NBC of 65.8 kJ mol^{-1} of H$^+$ extruded.

Note that in both Figs 4*B* and 5*B*, the ordinates refer to *net* acid-equivalent efflux through NHE and NBC respectively. The calculated efflux values were not corrected for possible simultaneous acid influx through sarcolemmal acid-loading transporters such as Cl$^-$–OH$^-$ exchange (CHE) and the Cl$^-$–HCO$_3^-$ anion exchanger (AE). Previous work at 37 °C has shown that acid loading through these latter two transporters is sufficiently small at pH$_i$ < 7.0 that it may conveniently be ignored (Leem *et al.* 1999).

Thermal sensitivity of resting pH$_i$

The trace shown in Fig. 6*A* demonstrates the typical effect on resting pH$_i$ of restoring the perfusion temperature from 27 to 37 °C under 5 % CO_2-buffered conditions (pH$_o$ 7.4). The middle trace shows that the raw SNARF ratio decreased within about 1 min, coinciding with the time course of rise of temperature in the bath (see Methods). Calibration of the ratiometric signal indicated that pH$_i$ became more acidic by 0.15 u within the 1 min period. Thereafter it remained constant for several minutes. On average, pH$_i$ fell by 0.13 u as shown in the histogram plotted in Fig. 6*B* (filled columns). Note that the discontinuity in the pH$_i$ trace in Fig. 6*A* corresponds to the time taken for the bath

Figure 3. Temperature dependence of intracellular intrinsic buffering

A, data pooled for intrinsic buffering measured at 37 °C (grey diamonds) and 27 °C (black diamonds) from cells prepulsed either with ammonium chloride or sodium acetate. Also shown (continuous grey line) is a previously determined relationship of the pH$_i$ dependence of intrinsic buffering at 37 °C (from Leem *et al.* 1999), calculated using eqn (1) in Methods. This was cross-checked against the sample measurements of β_i conducted at 37 °C (grey diamonds). For convenience, the continuous black line drawn through the points at 27 °C was fitted arbitrarily by a single buffer function (eqn (1)), concentration 36.5 mM and pK_a 6.83. At 27 °C, all data points averaged for n = 4–14, except at pH$_i$ 6.36 and 6.5 (each, n = 2), 6.58 and 7.82 (each, n = 1). At 37 °C, data points averaged for n = 3, except at pH$_i$ 7.32 and 7.05 (each, n = 2) and 6.76 (n = 1). Note that for n = 2, the upper and lower S.E.M. represent the individual data points. *B,* intracellular buffering capacity measured at 27 °C, expressed as a percentage of that computed at 37 °C, has been plotted *versus* pH$_i$. Over the whole pH$_i$ range tested, the mean reduction in buffering capacity during hypothermia is 40 % (indicated by horizontal dashed line).
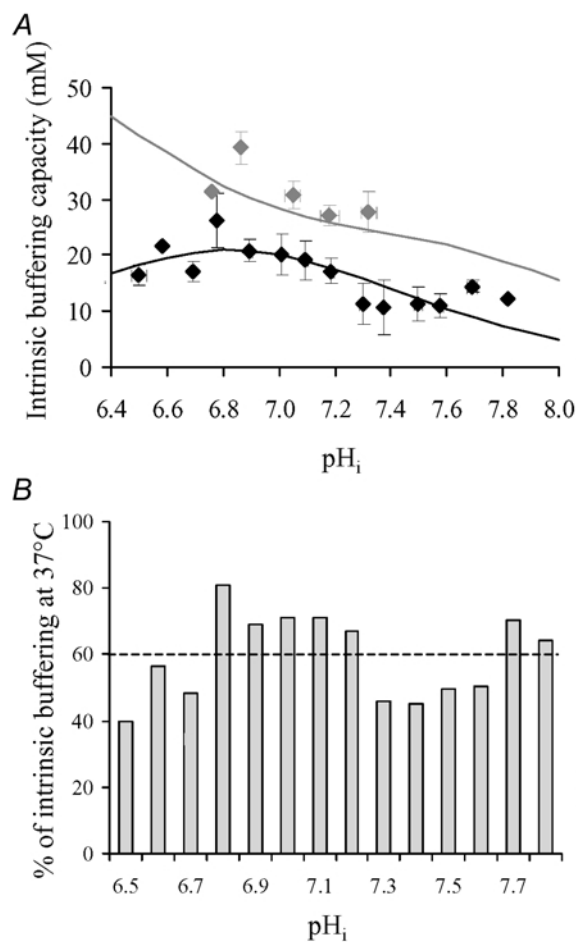
temperature to reach 37 °C (see above); given the temperature dependence of SNARF, the ratio could not be properly calibrated during this period. Quantitatively similar changes of resting pH_i occurred in Hepes-buffered conditions (effect not illustrated; pH_o 7.4). The mean pH_i values measured at the two temperatures are also plotted in Fig. 6*B* (open columns).

In another set of experiments, the thermally induced fall of pH_i (i.e. on raising temperature from 27 to 37 °C) was examined for various starting levels of pH_i, ranging from 7.35 to 6.45. This range was achieved partly by using cells not pre-pulsed with ammonium chloride where resting pH_i at 27 °C was 7.1–7.4, and partly by using cells pre-pulsed for varying intervals (3–6 min) with 15 mM ammonium

chloride in the presence of 30 μM Hoe 642. For these experiments, only Hepes-buffered superfusates were used. The results, pooled in Fig. 6*C*, show that the magnitude of the thermally induced acidosis was smaller at lower starting pH_i values and was absent at $pH_i \sim 6.45$.

DISCUSSION

The present work shows that activity of both $Na^+–H^+$ exchange (NHE) and $Na^+–HCO_3^-$ co-transport (NBC) in mammalian ventricular myocytes is strongly temperature dependent, a cooling of 10 °C reducing acid efflux by about 50 %. This is accompanied by a significant reduction of intrinsic buffering and a rise of resting pH_i.
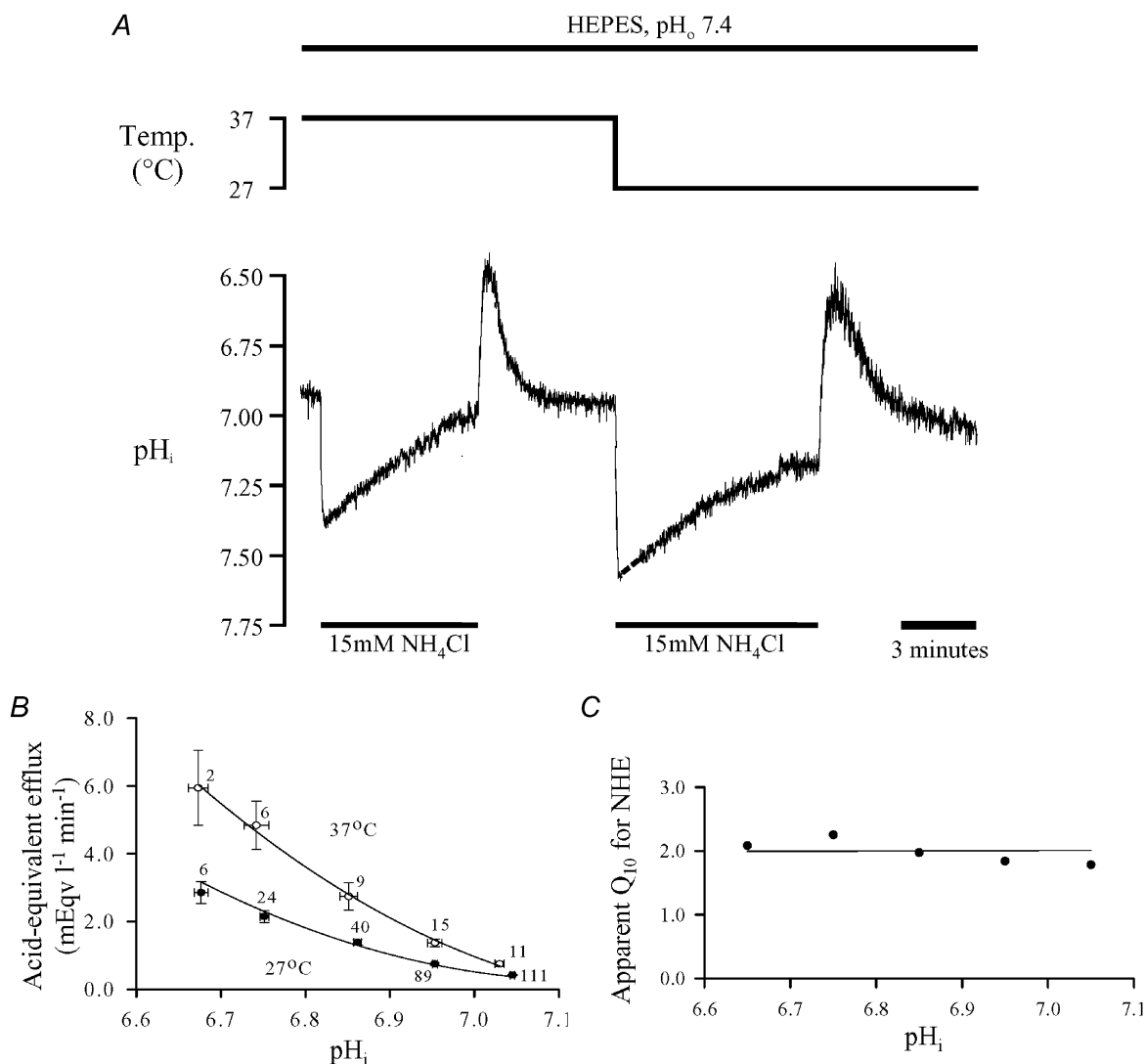

Figure 4. Temperature dependence of Na⁺–H⁺ exchange

A, following an acid load (induced by 15 mM NH₄Cl removal) in Hepes-buffered conditions, pH_i recovery was monitored, first at 37 °C and then again at 27 °C. Extracellular pH was 7.4 throughout. *B*, acid-equivalent efflux (mean ± S.E.M.), pooled from experiments similar to that shown in *A*, plotted as a function of pH_i at both 27 °C (●) and 37 °C (○). Numbers beside symbols denote the number of observations. *C*, apparent Q_{10} of Na⁺–H⁺ exchange, calculated as Q_{10} = (acid-equivalent efflux at 37 °C)/(acid-equivalent efflux at 27 °C) over the pH_i range shown in *A*.

Thermal sensitivity of intracellular buffering power
Intrinsic buffering. At 27 °C, β_i is about 60 % of that at 37 °C, as determined by ammonium and acetate prepulse techniques that have been validated extensively in cardiac cells (Vaughan-Jones & Wu, 1990; Bountra *et al.* 1990). In contrast, two other reports on reducing temperature in the heart have suggested a rise of intrinsic buffering; these were for a multicellular ventricular preparation *in vitro* (Do *et al.* 1996) and rat isolated ventricular myocytes (Hoshino & Avkiran, 2001). In non-cardiac tissues, either a decrease (Graber *et al.* 1992: opossum kidney cells) or no change (Aickin & Thomas, 1977*b*: skeletal muscle; Kiang *et al.* 1990: human A431 cells) has been reported.

Intrinsic buffering is attributable to the net effect of a range of constitutive, intracellular molecules, including taurine, amino acids, inorganic phosphate, proteins and dipeptides (e.g. homocarnosine, acetyl anserine). Many of these molecules contain histidine, the principal intracellular buffer (see e.g. Vaughan-Jones *et al.* 2002). Depending on which molecules are present, their concentrations and the temperature dependence of their respective pK_a values, the overall effect of temperature on total intrinsic buffering power may be expected to differ among cell types. This serves to emphasise that, for any given preparation, changes in β_i must be measured directly *in situ*. Values for β_i should, of course, be viewed with caution if plasma-
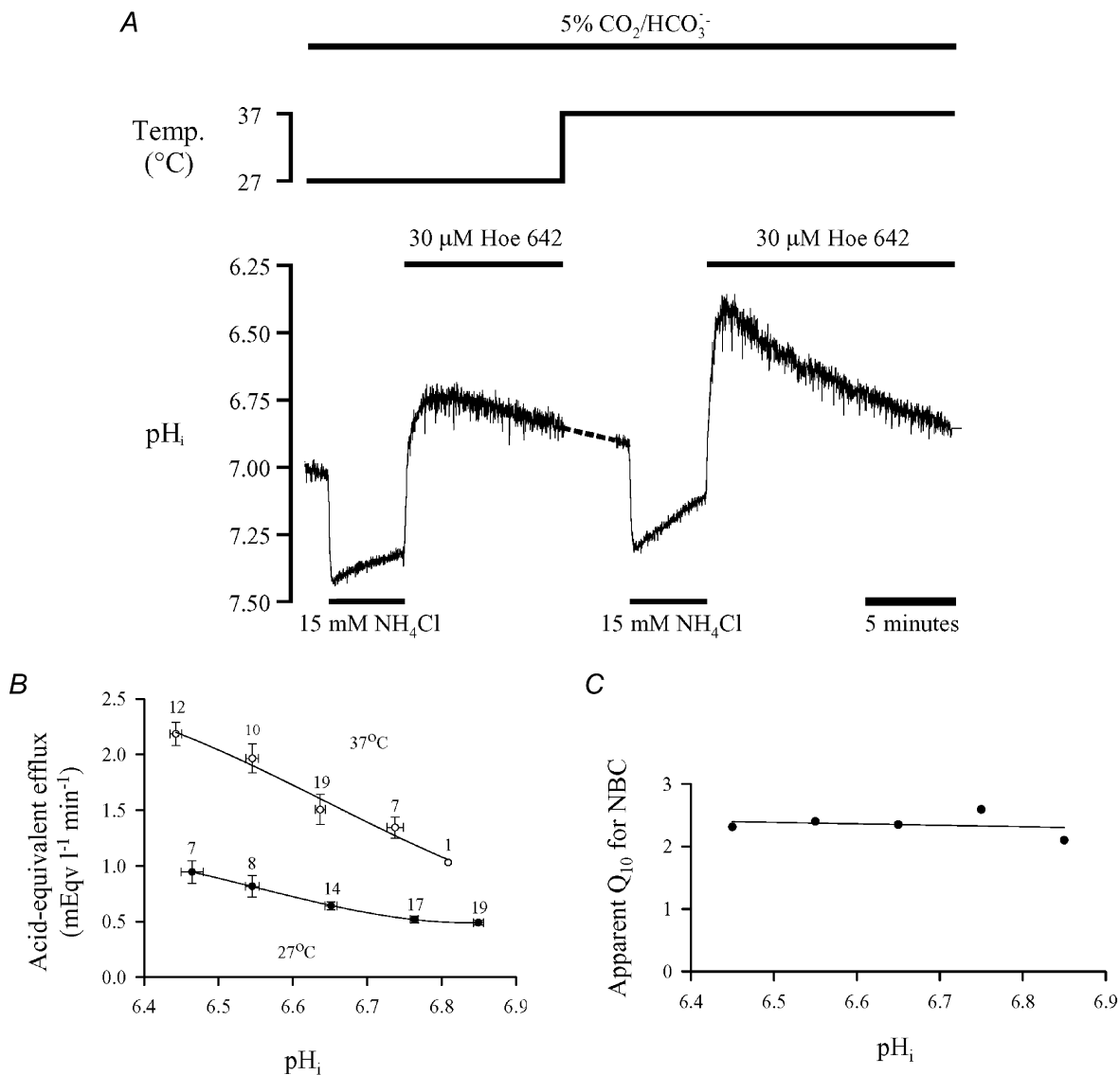

Figure 5. Temperature dependence of Na$^+$–HCO$_3^-$ co-transport
A, following an acid load (induced by 15 mM NH$_4$Cl removal) in 5 % CO$_2$/HCO$_3^-$-buffered conditions, pH$_i$ recovery was monitored, first at 37 °C and then again at 27 °C. Hoe 642 (30 μM) was added to inhibit Na$^+$–H$^+$ exchange and pH$_o$ was 7.35–7.40 throughout. *B,* acid-equivalent efflux (mean ± S.E.M.), pooled from experiments similar to that shown in *A*, plotted as a function of pH$_i$ at both 27 °C (●) and 37 °C (○). Numbers beside symbols denote the number of observations. *C,* apparent Q_{10} of Na$^+$–HCO$_3^-$ co-transport, calculated as Q_{10} = (acid-equivalent efflux at 37 °C)/(acid-equivalent efflux at 27 °C) over the pH$_i$ range shown in *A*.
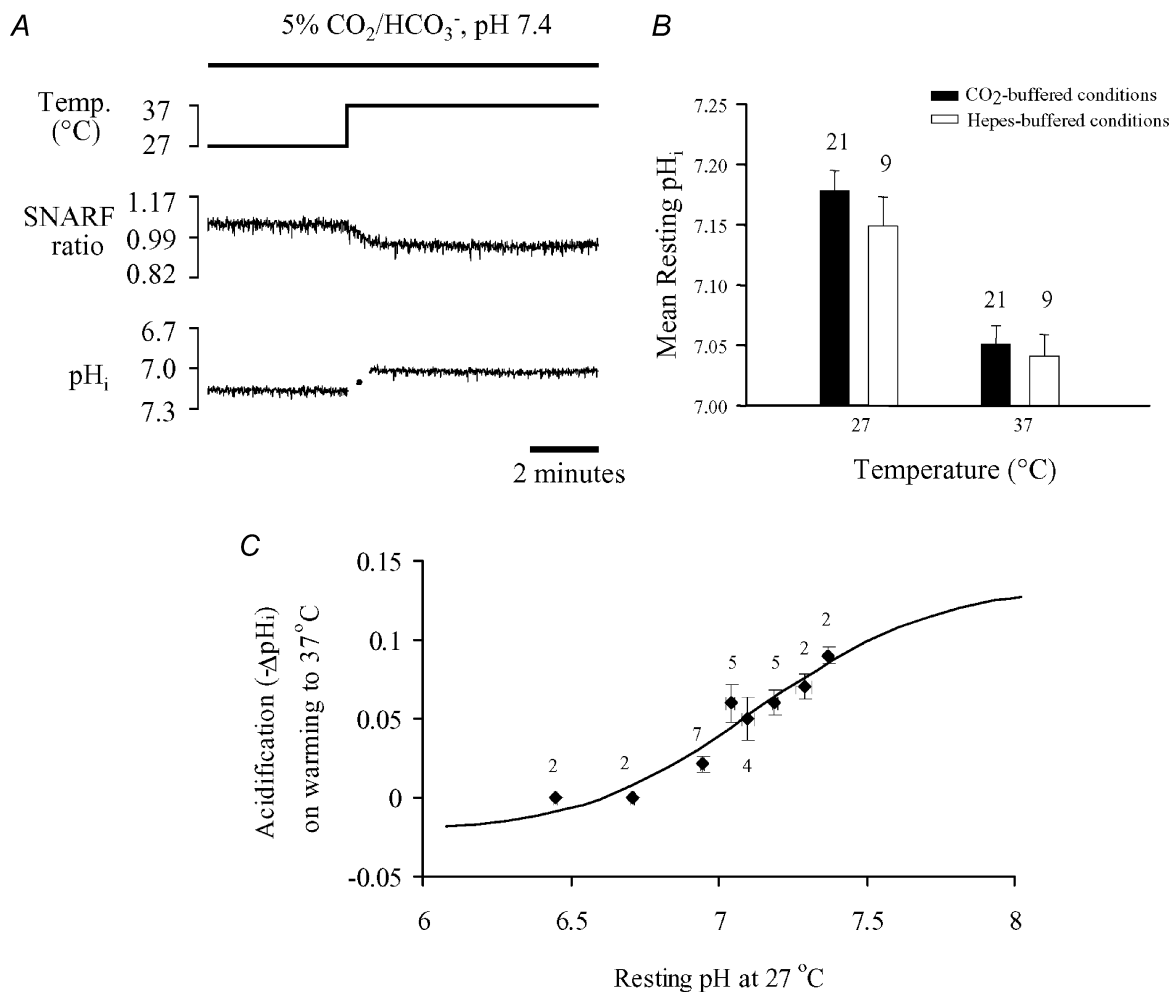
Figure 6. Temperature dependence of resting intracellular pH

A, effect of raising bath temperature from 27 to 37 °C on carboxy-SNARF-1 fluorescence ratio (middle trace) and corresponding pH_i (bottom trace) in 5 % CO_2/HCO_3^--buffered conditions, pH_o 7.35–7.40 at 37 °C. *B*, mean resting pH_i measured at 27 and 37 °C for 5 % CO_2/HCO_3^--buffered (filled columns) and Hepes-buffered (open columns) conditions. *C*, pooled data (mean ± S.E.M.) of the change in pH_i ($-\Delta pH_i$) following an increase in bath temperature from 27 to 37 °C in Hepes-buffered conditions. Numbers beside symbols denote the number of observations. Note that for $n = 2$, the upper and lower S.E.M. values represent the individual data points. Low initial pH_i levels were generated by pre-pulsing with 15 mM NH_4Cl in the presence of 30 μM Hoe 642 (see text for details). The continuous line shows predictions of the dual-buffer model described in the Appendix.
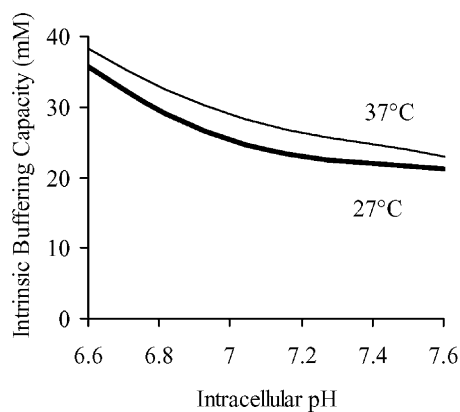

Figure 7. Modelling temperature dependence of intrinsic buffering power

The curve at 37 °C was computed from eqn (1) in Methods, i.e. for the sum of the two buffers, A and B, of concentration 84.22 and 29.38 mM respectively, and pK 6.03 and 7.57 respectively. The curve at 27 °C was computed for the same concentrations of A and B, but with pK_A = 6.005 and pK_B = 7.72.

lemmal acid extruders were active during the experimental measurements as this leads to an overestimate of β_i (Bountra *et al.* 1990). In recent temperature studies, however, (ferret, rat and guinea-pig heart; Do *et al.* 1996; Hoshino & Avkiran, 2001; and present work, respectively) acid extrusion via NHE was pharmacologically inhibited. It should also be noted that, at 37 °C, β_i appears very similar among ventricular myocytes from e.g. rat, rabbit and guinea-pig (Zaniboni *et al.* 2003). The cause of the different thermal sensitivity of ventricular β_i reported here for guinea-pig and that reported previously for ferret and rat therefore remains unexplained.

CO$_2$-dependent buffering. When computing total intracellular buffering power in 5 % CO$_2$-buffered conditions, a second component of intracellular buffering, β_{CO_2}, must be added to β_i. It can be shown that (see Methods):

$$\beta_{CO_2} = 2.303[HCO_3^-]_i = 2.303[HCO_3^-]_o \times 10^{(pH_i - pH_o)}. \quad (2)$$

Provided the cell remains an open system for CO$_2$, the value of β_{CO_2} at a given pH$_i$, $[HCO_3^-]_i$ and $[HCO_3^-]_o$ is thus temperature-insensitive.

Thermal sensitivity of NHE and NBC

Both NHE and NBC display a high temperature-sensitivity, their activities declining with temperature, indicating a Q_{10} of about 2 and 2.4, respectively. In other species and cell types, a Q_{10} for NHE of between 1.05 and 2.65 is evident (e.g. Aickin & Thomas, 1977*a*; Ives, 1985; Ellis & Macleod, 1985; Graber *et al.* 1992; Do *et al.* 1996; Hoshino & Avkiran, 2001). In some of these cases, however, Q_{10} values were determined from changes in dpH$_i$/dt rather than acid efflux (J^e), while in others they were determined without correcting for temperature effects on buffering power or the pH-sensitive fluorophore, or without allowing for significant contributions to J^e from NBC. In the present work we have attempted to account for these factors when computing the Q_{10} for NHE. In addition we have provided the first estimate for the Q_{10} of NBC.

The Q_{10} for NHE and NBC appears relatively constant over the pH$_i$ range (~6.60–7.50). This ensures that NHE and NBC operate optimally at 37 °C for a wide range of acid challenges. It is of interest that NHE activity has been shown to decrease on raising temperature above 40 °C (in A431 cells; Kiang *et al.* 1990; and in proximal tubule brush border membrane vesicles, Ives, 1985). This is consistent with mammalian proteins having an optimal operating temperature close to 37 °C. While protein denaturation is likely to be a factor at very high temperatures, modulation of NHE and NBC has also been suggested to occur through either an amphoteric amino acid on the transporter, e.g. histidine, which is selectively affected by the temperature change, or via an internal modulator such as a kinase that has temperature sensitivity (Graber *et al.* 1992).

Note that there are two reports of increased NHE activity on decreasing temperature below 37 °C. In guinea-pig erythrocytes, hypothermia stimulated amiloride-sensitive Na$^+$ uptake, suggested to be via NHE (Zhou & Willis, 1989); similar results were found in cultured chick embryonic myocytes using the NHE inhibitor EIPA (Knerr & Lieberman, 1993). The latter study, however, used neonatal cardiac cells that have unusually high NHE activity (Meno *et al.* 1989). From the present work, it is clear that in adult cells, hypothermia reduces both NHE and NBC activity.

Thermal sensitivity of resting pH$_i$

Resting pH$_i$ at 37 °C was similar in both Hepes- and CO$_2$-buffered conditions (7.04 and 7.05, respectively) and was comparable to that reported previously in guinea-pig myocytes (7.07 in Hepes and 7.04 in 5 % CO$_2$; Leem *et al.* 1999), as well as in ferret ventricle (7.03 in Hepes at 35 °C; Do *et al.* 1996). Resting pH$_i$ was ~0.1 pH units more alkaline at 27 °C but was again similar in the two buffer conditions.

Role of intrinsic buffer. The thermal decrease of pH$_i$ in guinea pig ventricular myocytes (27–37 °C) seems paradoxical given that NHE and NBC activity is enhanced on warming. Intuitively, a rise in pH$_i$ would be expected. One possibility is that temperature-elevation simultaneously stimulates sarcolemmal acid influx transporters (CHE and AE), thus effectively neutralising the influence on pH$_i$ of enhanced acid extrusion. An assessment of the temperature-sensitivity of acid loading transporters will be required to test this possibility. Effects on sarcolemmal acid–base transport, however, cannot account for the observed thermally induced fall of pH$_i$, as it is too rapid. Figure 6*A* shows that pH$_i$ falls by 0.15 u within 50 s of raising temperature by 10 °C. If caused by enhanced sarcolemmal acid-loading, this would be equivalent to an influx of 5 mM min^{-1} (influx = dpH$_i$/dt × β_i). At a pH$_i$ of 7.1 at 37 °C, CHE and AE each mediate an acid influx about 330-fold lower than this (~0.15 mM min^{-1}; Leem *et al.* 1999). The fall of pH$_i$ is thus likely to be mediated at intracellular buffer sites rather than at sarcolemmal transport sites.

A 10 °C rise in temperature acidifies most cells of most organisms by 0.1 to 0.2 pH units (Roos & Boron, 1981), including ectothermic and endothermic animals, hibernators and also plants (Marjanovic *et al.* 1998). Comparable changes in pH$_i$ have been found in mouse skeletal muscle (−0.0178 u °C^{-1}, Aickin & Thomas, 1977*a*; −0.015 u °C^{-1}, Westerblad *et al.* 1997), sheep Purkinje fibres (−0.015 u °C^{-1}; Ellis & Macleod, 1985), the right ventricle of ferret heart (−0.012 u °C^{-1}; Do *et al.* 1996), and Hepes-buffered rat myocytes (−0.015 u °C^{-1}; Hoshino & Avkiran, 2001). An effect of temperature on histidine residues, the primary intrinsic buffer, may partly underlie this phenomenon since, for a 10 °C rise, the pK_a of imidazole in aqueous

solution falls by 0.17 u (Reeves, 1972). This observation has led to the so-called 'alphastat hypothesis', where the fractional dissociation of imidazole histidine remains constant on changing temperature. As a consequence, ionic charge on intracellular proteins and other buffers is also proposed to be maintained (Reeves, 1972).

An intriguing observation is that thermal shifts of resting pH_i did not occur at pH_i 6.4 but were clearly evident at 7.4 (see Fig. 6C). The reason for this is not known, but one possibility is that the intrinsic buffers that dominate at pH_i 6.4 differ from those at 7.4, and that the former are less temperature sensitive. Leem et al. (1999) have provided evidence for at least two populations of intrinsic buffer in the ventricular myocyte, differing in their concentration and principal pK_a value (pK_a values of about 6.0 and 7.6 were suggested; see eqn (1) of present Methods). More recently, Zaniboni et al. (2003) have suggested that the low pK_a population comprises intracellular proteins, the remaining population comprising a mixture of lower molecular weight buffers with higher pK_a values, such as dipeptides and inorganic phosphate. In the Appendix to the present paper we show that a simple model that utilises the above two categories of buffer predicts a thermally induced acidification of resting pH_i ($-\Delta pH_i$), accompanied by an increase of β_i, as observed experimentally. The model indicates that the $-\Delta pH_i$ is consistent with an average 0.15 unit fall in pK_a for the higher-pK_a buffers, with little or no change in the lower-pK_a population. The predictions for $-\Delta pH_i$ have been included (continuous line) with the experimental measurements plotted in Fig. 6C.

The predictive behaviour of the dual-buffer model strongly suggests that a heterogeneous population of intracellular intrinsic buffers displaying different temperature-sensitive pK_a values may account for the thermal sensitivity of resting pH_i and intracellular buffering power. Given that most intrinsic buffering is attributable to imidazole histidine, the above hypothesis implies that the microenvironment around such groups on, say, intracellular proteins (the proposed lower-pK_a category of buffer; Zaniboni et al. 2003) may possibly confer a lower thermal sensitivity on pK_a than that for imidazole located on dipeptides such as homocarnosine and acetylanserine that have been proposed to comprise the higher-pK_a category.

Role of carbonic buffer. Thermal intracellular acidosis is the same in the presence and absence of CO_2/HCO_3^-. While the explanation for this is not entirely clear, it is notable that the pH of a 5 % CO_2/22 mM HCO_3^--buffered Tyrode solution is largely temperature-insensitive: its pH falls from 7.40 to 7.35 on cooling from 37 to 27 °C. This lack of major temperature sensitivity can be attributed to a rise in the pK_a of carbonic acid at lower temperature being

offset by an increase in the solubility of CO_2 (Seldin & Giebisch, 1989). Thermal sensitivity of pH_i therefore depends largely on intrinsic rather than carbonic buffer.

Hypothermia and cardiac physiology and pathophysiology

Low temperature experiments. The present findings reinforce the need to interpret results of experiments performed at low temperature with some caution. For example, the amplitude of rapid cooling contractures associated with release Ca^{2+} from the sarcoplasmic reticulum (e.g. Bers, 1987) may in part be due to an alkaline shift on cooling, as intracellular alkalosis increases myofilament Ca^{2+}-sensitivity (Fabiato & Fabiato, 1978). In the case of protocols performed at room temperature, pH_i regulation will be slowed considerably, and so manoeuvres that displace pH_i will produce larger and more sustained effects. Given the important link between H_i^+ and Ca_i^{2+} in heart, such abnormalities in pH_i control will be expected to distort the normal physiological control of Ca_i^{2+}.

Myocardial ischaemia and reperfusion. The thermal sensitivity of NHE and NBC may be relevant to cardiac injury and arrhythmogenesis during pathophysiological conditions. Na^+ influx coupled to acid efflux plays an important role in the generation of Na_i^+-driven Ca_i^{2+} overload during conditions of low pH_i, such as those found during ischaemia and early post-ischaemic reperfusion. Intracellular Ca^{2+} overload impairs cardiac function (e.g. Piper et al. 1996) and may be arrhythmogenic (e.g. Ch'en et al. 1998). In animal and whole heart models of ischaemia, pharmacological NHE inhibitors such as Hoe 694 and cariporide (Hoe 642) that suppress Na^+-coupled acid efflux appear to reduce the incidence of reperfusion injury and arrhythmia, and are thus potentially useful cardioprotective agents (Karmazyn, 1999; Hartmann & Decking, 1999; Xiao & Allen, 2000). Hypothermia offers another potential cardioprotective route as it decreases not only NHE (Hoshino & Avkiran, 2001), but also NBC activity, both of which are sources of Na^+ influx. Notably, hypothermia (4–10 °C) is often combined with cardioplegia to minimise myocardial damage during cardiac surgery. If the high Q_{10} of the transporters extends to these low temperatures, then they will operate at about 10 % of their rate at 37 °C. Na^+ influx during acid overload, such as that associated with post-ischaemic reperfusion, would thus be severely attenuated, especially when combined with pharmacological NHE inhibitors; indeed this strategy appears to offer additional cardioprotection during reperfusion in animals (Shipolini et al. 1997). In conclusion, the thermal sensitivity of NHE and NBC is consistent with suggestions that inhibition of these transporters may contribute to some of the cardioprotective effects of cooling during ischaemia and reperfusion.

APPENDIX

Modelling the thermal sensitivity of pHi and intracellular intrinsic buffering

For simplicity, intracellular, intrinsic buffering capacity is assumed to be a function of the concentration and dissociation constant (K) of two populations of buffer (A and B), as suggested previously (Leem *et al.* 1999; Zaniboni *et al.* 2003). A change of temperature is assumed to affect K only (e.g. Reeves, 1972; Seldin & Giebisch, 1989). Assuming the total concentration of intracellular buffer remains constant, numerical minimalisation algorithms (fminsearch, MATLAB, Mathworks Corporation) may then be used to find the equilibrium concentrations of H^+, and the protonated and unprotonated forms of the buffers, after a change in their dissociation constants.

We assume a temperature dependence of the dissociation of water, K_w, such that the ratio, γ,

$$\gamma = \sqrt{\frac{K_w^{37°C}}{K_w^{27°C}}} \approx 1.36.$$

Next we define a function f that must equal zero at equilibrium,

$$f(\delta_1, \delta_2) = \left(\frac{\left(\frac{H_0}{\gamma} + \delta_1 + \delta_2\right) \cdot (A_0 + \delta_1)}{HA_0 - \delta_1} - K_A \right)^2 + \left(\frac{\left(\frac{H_0}{\gamma} + \delta_1 + \delta_2\right) \cdot (B_0 + \delta_2)}{HB_0 - \delta_2} - K_B \right)^2$$

where H_0, A_0, HA_0, B_0 and HB_0 are the initial concentrations of protons and the unprotonated and protonated forms of buffers A and B, before the temperature change. Constants K_A and K_B are the dissociation constants for the two buffers at the new temperature. We minimise f with respect to variables δ_1 and δ_2. New equilibrium concentrations are given as:

$$H = H_0/\gamma + \delta_1 + \delta_2$$

$$A = A_0 + \delta_1 \qquad HA = HA_0 - \delta_1$$

$$B = B_0 + \delta_2 \qquad HB = HB_0 - \Delta_2.$$

We then use data from Fig. 6*C* to quantify the temperature dependence of K_A and K_B. We assume the values for K_A and K_B determined previously at 37 °C (pK values of 6.03 and 7.57 respectively; Leem *et al.* 1999). Least squares fitting suggests that the pHi dependence of the acidosis during warming from 27 to 37 °C ($-\Delta$pHi) can be reproduced if pK_A is slightly lower at 27 °C (6.005), and pK_B is higher (7.72) than at 37 °C. This latter 0.15 u shift is similar to that reported previously for the temperature sensitivity of imidazole (Reeves, 1972). The pHi sensitivity of $-\Delta$pHi predicted by these changes of K_A and K_B has been superimposed (continuous line) on the experimental data shown in Fig. 6*C*.

Changes in K_A and K_B will alter the pHi sensitivity of total intrinsic buffering power (β_i). At any given pHi, intracellular buffering equals the sum ($\beta_A + \beta_B$) of buffer capacities for A and B. The thin line in Fig. 7 shows total β_i *vs.* pHi determined

previously at 37 °C by Leem *et al.* (1999). The thick line shows the relationship at 27 °C, predicted from the K_A and K_B values at this temperature (relationship calculated using eqn (1) in Methods). It is notable that over the pHi range 7.6–6.6, total β_i is significantly lower at 27 °C than at 37 °C, in agreement with the experimentally observed decrease of β_i during hypothermia (Fig. 3). The predicted decrease is, however, ~20 % rather than the 40 % decrease actually measured, suggesting that the dual buffer model is not entirely successful in its predictions. Nevertheless, the model illustrates well the principle that the observed rise of resting pHi and fall of intrinsic buffering capacity during hypothermia may occur as a result of increases in the pK of selected intracellular buffers.

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