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Effects of moderate hypothermia on sarcolemmal Na^+/H^+ exchanger activity and its inhibition by cariporide in cardiac ventricular myocytes

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1 Specific inhibitors of the sarcolemmal Na^+/H^+ exchanger (NHE) such as cariporide are being evaluated for cardioprotective therapy during cardiac surgery. We determined the effects of moderate hypothermia (25°C), as occurs during cardiac surgery, on (1) sarcolemmal NHE activity and (2) the NHE-inhibitory potency of cariporide, in isolated adult rat ventricular myocytes.

2 As the index of NHE activity, trans-sarcolemmal acid efflux rate (J_H) was determined by microepifluorescence in single cells ($n=8$ to 11 per group), during recovery from intracellular acidosis in bicarbonate-free conditions.

3 Initially, myocytes were subjected to two consecutive acid pulses; these both occurred at 37°C in the normothermic control group but the second pulse was at 25°C in the moderate hypothermia group. J_H values obtained after the first pulse were superimposed in both groups, indicating comparable cell populations. However, after the second pulse, J_H values in the moderate hypothermia group were approximately 50% of those in the normothermic control group over the pH_i range 6.80–7.10.

4 Similar results were obtained in cells subjected to a single acid pulse at 37 or 25°C, with J_H values in the latter group measuring approximately 60% of those in the former over the pH_i range 6.80–7.10.

5 Cariporide (0.01, 0.03, 0.1, 0.3, 1.0 or 3.0 μM), present during recovery from a single acid pulse, reduced J_H in a concentration-dependent manner, with IC_{50} values of 150 and 130 nM at 37 and 25°C, respectively.

6 We conclude that moderate hypothermia produces (1) a significant, but partial, inhibition of sarcolemmal NHE activity, and (2) no significant effect on the NHE-inhibitory potency of cariporide.

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Abbreviations: β_i, intrinsic buffering power; cSNARF-1, carboxy-seminaphthorhodafluor-1; GUARDIAN, Guard During Ischaemia Against Necrosis trial; HOE-642, 4-isopropyl-3-methylsulphonylbenzoyl-guanidine methanesulphonate; J_H, rate of acid efflux; NHE, Na^+/H^+ exchange; pH_i, intracellular pH

Introduction

There is substantial pre-clinical evidence that recently-developed, specific Na^+/H^+ exchanger (NHE) inhibitors protect the myocardium during ischaemia and reperfusion (see reviews by Avkiran (1999b) and Karmazyn *et al.* (1999)). Indeed, the degree of protection afforded by NHE inhibition appears to be at least as good as that afforded by ischaemic preconditioning (Avkiran, 1999a; Gumina *et al.*, 1999; Shipolini *et al.*, 1997b). In the vast majority of pre-clinical studies with NHE inhibitors, hearts have been subjected to ischaemia and reperfusion under normothermic conditions, in an attempt to mimic the situation that occurs during spontaneous coronary occlusion and subsequent revascularization in patients with coronary artery disease. Nevertheless, a few studies (Kim *et al.*, 1998a, b; Myers & Karmazyn, 1996; Shipolini *et al.*, 1997a; Yamauchi *et al.*, 1997) have employed global hypothermic ischaemia, as encountered during cardiac surgery and transplantation, and have used NHE inhibitors in combination with established surgical cardioprotection techniques (such as hyperkalaemic cardioplegic arrest), with encouraging findings. For example, our laboratory was the first to show that the specific NHE inhibitor cariporide (HOE-642; 4-isopropyl-3-methylsulphonylbenzoyl-guanidine methanesulphonate), used as an adjunct or additive to crystalloid cardioplegia, provides additional cardioprotective benefit under conditions of both moderate hypothermia (28°C), as encountered during routine cardiac surgery, and severe hypothermia (7.5°C), as used for cardiac preservation for transplantation (Shipolini *et al.*, 1997a). Interestingly, data from the GUARDIAN trial (Théroux *et al.*, 2000) indicate that a subgroup of high-risk patients subjected to iatrogenic myocardial ischaemia during coronary artery bypass graft surgery uniquely benefited from treatment with cariporide.

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Its common occurrence under hypothermic conditions is a potentially important factor that distinguishes iatrogenic myocardial ischaemia during cardiac surgery from myocardial ischaemia that manifests during spontaneous coronary events. Despite the experimental work that has been carried out to determine the cardioprotective efficacy of NHE inhibitors under conditions of normothermic and hypothermic ischaemia, however, the effects of reduced temperature *per se* on sarcolemmal NHE activity and on the potency of NHE inhibitors have not been fully characterized. Therefore, the objectives of the work described here were to determine, in adult rat ventricular myocytes, the effects of moderate hypothermia (25°C) on (1) sarcolemmal NHE activity and (2) the NHE-inhibitory potency of cariporide.

Methods

This investigation was performed in accordance with the Home Office 'Guidance on the Operation of the Animals (Scientific Procedures) Act 1986', published by Her Majesty's Stationery Office, London.

Isolation of ventricular myocytes

Adult male Wistar rats (200–250 g) were anaesthetized with sodium pentobarbitone (60 mg kg^{-1} i.p.) and injected with heparin (50 u i.v.), and their hearts were excised for the isolation of ventricular myocytes by enzymatic digestion, as described previously (Yasutake *et al*., 1996).

Measurement of pH_i

Intracellular pH (pH_i) was monitored in single myocytes loaded with the pH-sensitive fluoroprobe carboxy-semi-naphthorhodafluor-1 (cSNARF-1), using an established microepifluorescence technique (Avkiran & Yokoyama., 2000; Gunasegaram *et al*., 1999; Haworth *et al*., 1997; 1999; Snabaitis *et al*., 2000; Yasutake *et al*., 1996; Yokoyama *et al*., 1998; 2000). Calibration of the cSNARF-1 signal was carried out *in situ* at both 37°C (13 cells) and 25°C (10 cells) using nigericin-containing calibration solutions, as described in detail previously (Yasutake *et al*., 1996). Also as described previously (Yasutake *et al*., 1996), at the end of each experiment, myocytes were exposed to the pH 7.0 calibration solution and the normalized fluorescence emission ratios were converted to pH_i values by reference to a calibration curve that was obtained by a nonlinear least-squares fit of data to the equation given below.

$$\frac{I_{580}/I_{640}}{(I_{580}/I_{640})_{pH7}} = 1 + a\left[\frac{10^{(pH-pK)}}{1+10^{(pH-pK)}} - \frac{10^{(7-pK)}}{1+10^{(7-pK)}}\right]$$

Estimation of intracellular intrinsic buffering power

Intracellular intrinsic buffering power (β_i) was estimated by stepwise removal of extracellular NH_4Cl at both 37°C (10 cells) and 25°C (11 cells), as described in detail previously (Yasutake *et al*., 1996). At each step, calculated changes in $[NH_4^+]_i$ and measured changes in pH_i were used to estimate β_i, from the equation $\beta_i = [NH_4^+]_i/pH_i$.

Determination of sarcolemmal NHE activity

The myocytes were maintained in bicarbonate-free Tyrode's solution throughout each experiment, thus enabling the rate of acid efflux (J_H), which was calculated from the equation $J_H = \beta_i \cdot dpH_i/dt$ (where dpH_i/dt is the rate of recovery of pH_i), to be used as an indicator of sarcolemmal NHE activity (Yasutake *et al*., 1996). J_H values were determined either at pH_i intervals of 0.05 throughout recovery from intracellular acidosis (when studying the effects of temperature) or during the first 60 s after the induction of intracellular acidosis (when studying the effects of cariporide).

Experimental protocols

In initial studies, myocytes ($n=9$ or 10 per group) were subjected to intracellular acidosis by transient exposure to 20 mM NH_4Cl and its subsequent washout for 8 min (first acid pulse), which was repeated 10–12 min later (second acid pulse) (Avkiran & Yokoyama., 2000; Gunasegaram *et al*., 1999; Snabaitis *et al*., 2000; Yasutake *et al*., 1996; Yokoyama *et al*., 1998). In both normothermic control and moderate hypothermia groups, the first acid pulse occurred at 37°C and was induced by a 4 min exposure to NH_4Cl. In the normothermic control group, cells were maintained at 37°C throughout the experiment and the second acid pulse was induced under identical conditions to the first. In contrast, in the moderate hypothermia group, cells were switched to superfusion at 25°C from 10 min before the second acid pulse and were maintained at this temperature thereafter; in this group, the second acid pulse was induced by a 6 min exposure to NH_4Cl. In subsequent experiments, cells ($n=8$ to 11 per group) were subjected to a single acid pulse at either 37 or 25°C by transient (4 min at 37°C, 6 min at 25°C) exposure to 20 mM NH_4Cl and its subsequent washout for 8 min; when used, cariporide (0.01, 0.03, 0.1, 0.3, 1.0 or 3.0 μM) was present throughout NH_4Cl washout.

Data analysis

Data are expressed as mean ± s.e.mean. Experiments within each protocol were randomized. For inter-group comparisons of data, either analysis of variance (for multi-group comparisons) or the unpaired *t*-test (for comparisons between normothermic control and moderate hypothermia groups) was used. $P<0.05$ was considered significant. Dose-response curves and IC_{50} values were obtained by nonlinear regression analysis, using GraphPad Prism software.

Results

Effects of moderate hypothermia on cSNARF-1 calibration

The *in situ* calibration curves obtained at 37 and 25°C are shown in Figure 1. As illustrated, moderate hypothermia altered the pH_i-dependence of the fluorescence emission ratio

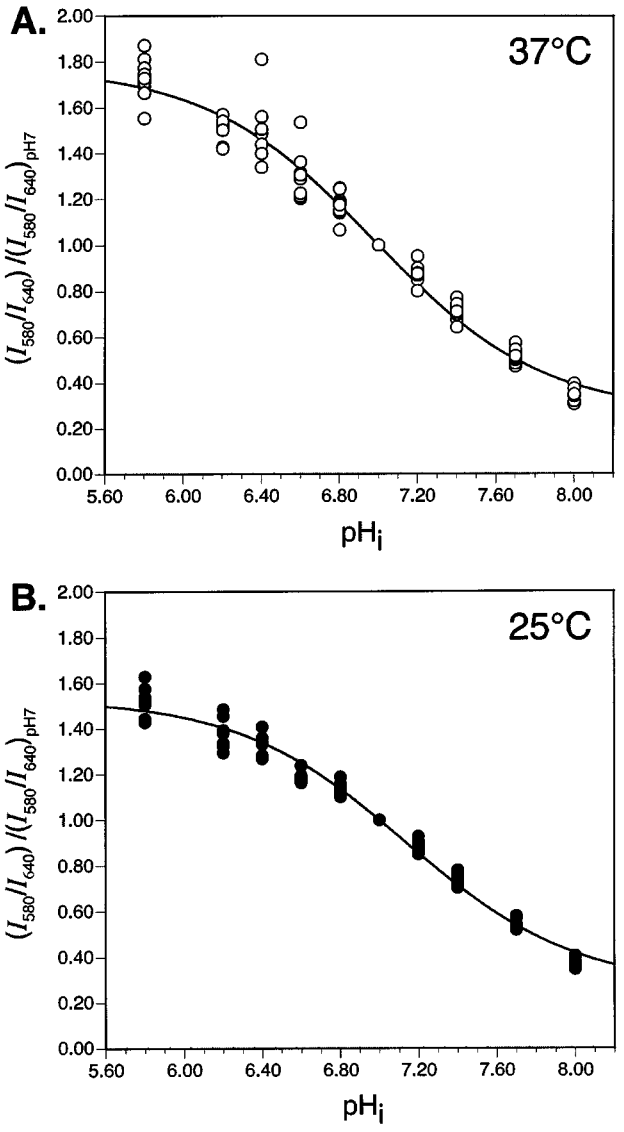

Figure 1 Calibration curves constructed using normalized I_{580}/I_{640} ratio data obtained during exposure of adult rat ventricular myocytes to nigericin-containing calibration solutions at (A) 37°C ($n=13$ cells) or (B) 25°C ($n=10$ cells). See text for details.

of cSNARF-1, with estimated pK values for the fluoroprobe of approximately 7.00 at 37°C and 7.15 at 25°C. A comparable temperature-dependent pK change has been reported previously for another pH-sensitive fluoroprobe, 2 7-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (Graber *et al.*, 1992). In subsequent experiments, fluorescence emission ratios were converted to pH_i values by reference to the calibration curve obtained at the appropriate temperature (Figure 1).

Effects of moderate hypothermia on β_i

As shown in Figure 2, moderate hypothermia produced a small change in the pH_i-dependence of β_i, such that β_i tended to be greater at 25°C than at 37°C, particularly under non-acidic conditions. For the calculation of J_H in subsequent experiments, β_i was estimated by reference to the β_i-versus-pH_i relationship obtained at the appropriate temperature (Figure 2).

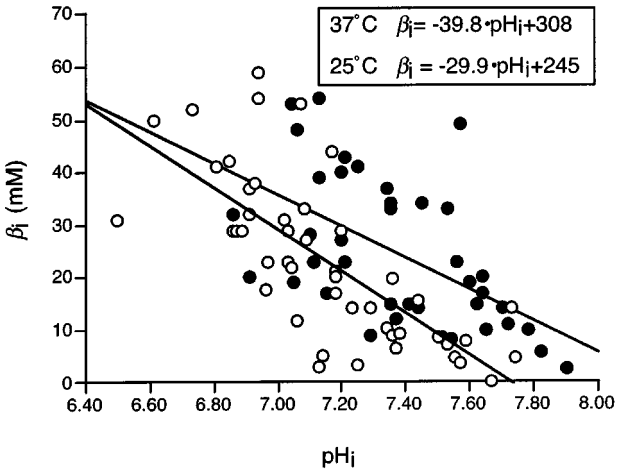

Figure 2 The relationship between intracellular pH (pH_i) and intrinsic buffering power (β_i) in adult rat ventricular myocytes at 37°C ($n=10$ cells, open symbols) and 25°C ($n=11$ cells, solid symbols). Inset shows the equations obtained by linear regression analysis of the data obtained at each temperature.

Effects of moderate hypothermia on sarcolemmal NHE activity

In the first set of experiments to determine the effect of moderate hypothermia on sarcolemmal NHE activity, two groups of cells were subjected to consecutive acid pulses, after each of which NHE activity was determined. The first pulse was at 37°C in both groups (to confirm comparable cell populations) while the second pulse was at either 37°C (normothermic control group, $n=10$) or 25°C (moderate hypothermia group, $n=9$). Basal pH_i values at 37°C, obtained prior to the first acid pulse, were comparable in the normothermic control and moderate hypothermia groups, measuring 7.31 ± 0.02 and 7.28 ± 0.04, respectively (NS). Cells in the normothermic control and moderate hypothermia groups acidified to a similar extent during the first acid pulse at 37°C, with minimum pH_i values of 6.62 ± 0.03 and 6.64 ± 0.07, respectively; during the second acid pulse, however, the minimum pH_i was 6.66 ± 0.03 in the normothermic control group but tended to be higher at 6.76 ± 0.06 in the moderate hypothermia group. Figure 3 shows the J_H-versus-pH_i curves obtained after both acid pulses in the two study groups. NHE activity during recovery from the first acid pulse at 37°C was similar in both groups, with comparable J_H values obtained throughout the pH_i range 6.80–7.10 (Figure 3A). For example, J_H at pH_i 6.90 was 3.79 ± 0.75 mM min^{-1} in the normothermic control group and 3.78 ± 0.43 mM min^{-1} in the moderate hypothermia group (NS). In contrast, after the second acid pulse (which was carried out at 37°C in the normothermic control group but at 25°C in the moderate hypothermia group), J_H in the moderate hypothermia group measured approximately 50% of that seen in the normothermic control group throughout the pH_i range 6.80–7.10 (Figure 3B). This difference between the groups arose predominantly from an increase in NHE activity between the two acid pulses in the normothermic

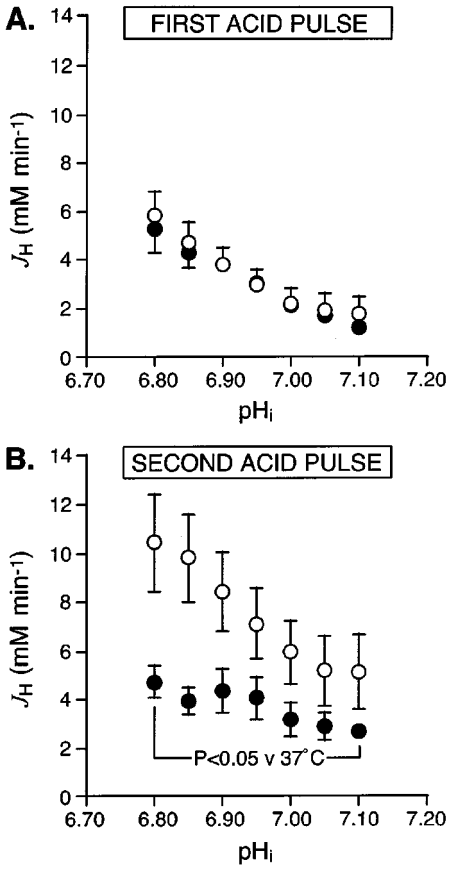

Figure 3 Effect of moderate hypothermia on sarcolemmal NHE activity in adult rat ventricular myocytes subjected to two consecutive acid pulses. Figure shows the J_H-versus-pH$_i$ curves obtained during (A) the first acid pulse and (B) the second acid pulse. In the normothermic control group ($n=10$ cells, open symbols) both acid pulses occurred at 37°C, whereas in the moderate hypothermia group ($n=9$ cells, solid symbols) cells were switched from 37 to 25°C from 10 min before the second acid pulse. The curves were constructed by determining J_H values at pH$_i$ intervals of 0.05 in each cell, throughout recovery from both acid pulses.

control group but not in the moderate hypothermia group. It appears therefore that consecutive acid pulses produce an increase in sarcolemmal NHE activity in adult rat ventricular myocytes under normothermic conditions and that this increase is inhibited by exposure of the cells to moderate hypothermia.

To determine the effect of moderate hypothermia on NHE activity in the absence of any changes arising from repeated episodes of intracellular acidosis, we next examined NHE activity in two groups of cells ($n=8$ or 9 per group) subjected to a single acid pulse, at either 37 or 25°C. In these experiments, the basal pH$_i$ value obtained prior to the acid pulse was 7.32 ± 0.07 in the normothermic control group ($n=9$) but was significantly higher at 7.46 ± 0.03 in the moderate hypothermia group ($n=8$). The minimum pH$_i$ achieved during the acid pulse was also significantly higher in the moderate hypothermia group (6.83 ± 0.03) relative to the normothermic control group (6.67 ± 0.04). Figure 4 shows the J_H-versus-pH$_i$ curves obtained in both study groups; as can be seen, the curve was shifted to the left under conditions of moderate hypothermia, reflecting significantly lower NHE activity

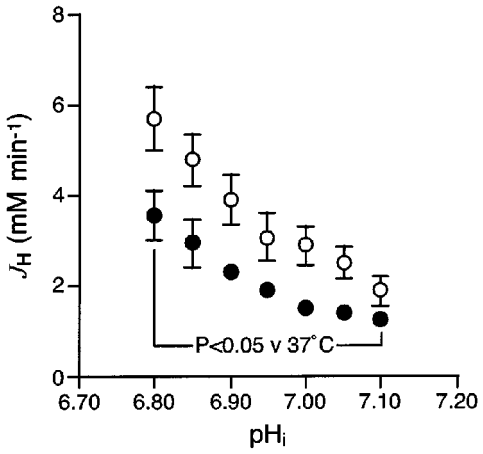

Figure 4 Effect of moderate hypothermia on sarcolemmal NHE activity in adult rat ventricular myocytes subjected to a single acid pulse. Figure shows the J_H-versus-pH$_i$ curves for the normothermic control group ($n=9$ cells, open symbols), in which the acid pulse occurred at 37°C, and the moderate hypothermia group ($n=8$ cells, solid symbols), in which the acid pulse occurred at 25°C. The curves were constructed by determining J_H values at pH$_i$ intervals of 0.05 in each cell, throughout recovery from the acid pulse.

throughout the pH$_i$ range 6.80–7.10. For example, J_H at pH$_i$ 6.90 was 3.91 ± 0.55 mM min^{-1} in the normothermic control group but measured only 59% of that, at 2.32 ± 0.24 mM min^{-1}, in the moderate hypothermia group ($P<0.05$).

Effects of moderate hypothermia on the NHE-inhibitory potency of cariporide

In this protocol, we sought to determine whether the NHE-inhibitory potency of cariporide is altered under conditions of moderate hypothermia. Twelve groups of cells ($n=8$ to 11 per group) were again subjected to a single acid pulse at either 37 or 25°C, with cariporide (0.01–3.0 μM) present in the superfusion solution throughout the recovery phase. Since cariporide inhibits recovery from intracellular acidosis, J_H was determined only at the nadir of the acid pulse, as illustrated in Figure 5. The basal pH$_i$ value obtained prior to the acid pulse was 7.25 ± 0.01 in the cells studied at 37°C ($n=52$) and again was significantly higher at 7.45 ± 0.02 in the cells studied at 25°C ($n=53$). The minimum pH$_i$ value, obtained upon NH_4Cl washout, was 6.64 ± 0.02 in the cells studied at 37°C ($n=52$) and was also significantly higher at 6.72 ± 0.02 in the cells studied at 25°C ($n=53$). At each temperature, however, there was no significant difference in either the basal pH$_i$ or the minimum pH$_i$ between the six groups that received the different concentrations of cariporide (Table 1).

Figure 6A shows the J_H values obtained in the various study groups at 37 and 25°C. As can be seen, in the presence of each concentration of cariporide, the J_H obtained at 25°C was approximately 50–60% of that obtained at 37°C, which is consistent with our observations above. At both temperatures, cariporide produced a concentration-dependent reduction in J_H (Figure 6A). Notably, even in the presence of 3.0 μM cariporide, J_H was not completely abolished, most likely due to residual Na^+/HCO_3^- cotransport activity (Wu

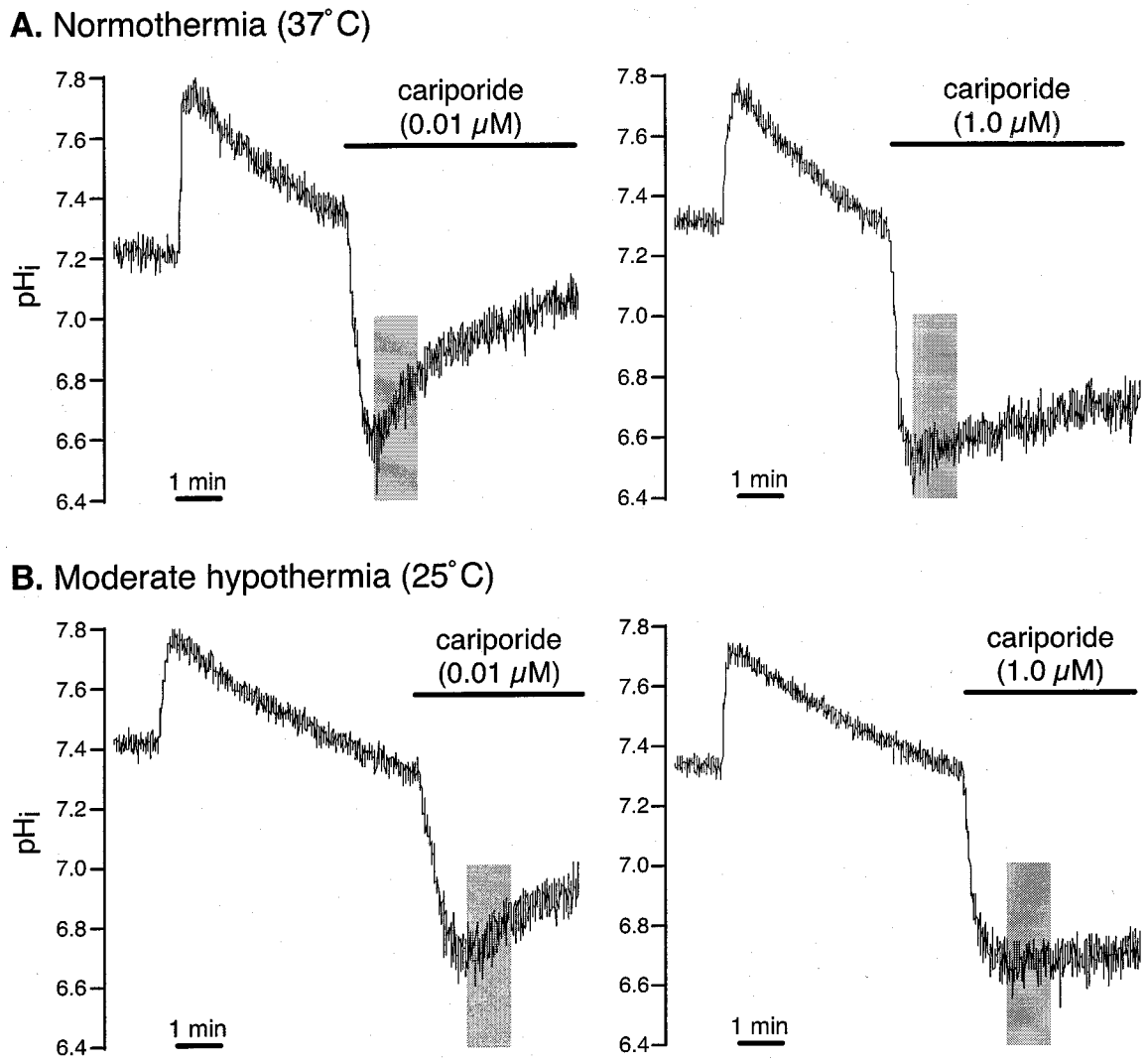

Figure 5 Effect of cariporide on recovery from intracellular acidosis in adult rat ventricular myocytes. Figure shows representative pH_i recordings obtained in cells exposed to a low, non-inhibitory concentration (0.01 μM) or a high, inhibitory concentration (1.0 μM) of cariporide during acid pulses carried out under conditions of (A) normothermia (37°C) or (B) moderate hypothermia (25°C). Shaded areas indicate the period during which J_H was estimated from the rate of recovery of pH_i.

Table 1 Basal and minimum pH_i values in cells exposed to cariporide at 37 or 25°C.

Cariporide (μM)	n	*37°C* *basal pH_i*	*min pH_i*	n	*25°C* *basal pH_i*	*min pH_i*
0.01	9	7.23±0.03	6.62±0.03	9	7.44±0.03	6.78±0.03
0.03	8	7.23±0.03	6.66±0.06	9	7.51±0.04	6.75±0.05
0.1	9	7.25±0.04	6.59±0.03	8	7.41±0.04	6.68±0.06
0.3	8	7.29±0.04	6.70±0.04	8	7.47±0.06	6.74±0.04
1.0	9	7.31±0.03	6.67±0.03	11	7.45±0.04	6.72±0.03
3.0	9	7.22±00.3	6.58±0.02	8	7.43±0.03	6.66±0.03

For each group, *n* indicates the number of cells studied. In each cell, basal and minimum pH_i values were noted immediately before exposure to and immediately after washout of NH_4Cl, respectively.

et al., 1994). Figure 6B illustrates the dose-response curves for NHE inhibition by cariporide, obtained after the correction of J_H values for residual Na^+/HCO_3^- cotransport activity, at both 37 and 25°C. As can be seen, moderate hypothermia did not have a significant effect on the NHE-inhibitory potency of cariporide, with IC_{50} values of approximately 150 nM at 37°C and 130 nM at 25°C.

Discussion

Although several studies have attempted to determine the effects of moderate hypothermia (20–30°C) on plasma membrane NHE activity, their findings have been somewhat contradictory, probably due to the variety of cell types that have been used and the manner in which NHE activity has

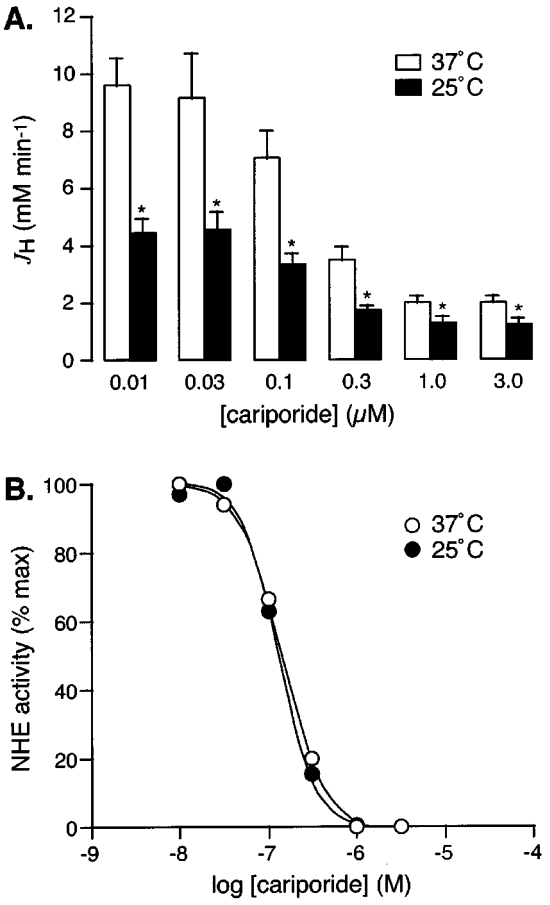

Figure 6 Effect of moderate hypothermia on the NHE-inhibitory potency of cariporide in adult rat ventricular myocytes. Figure shows (A) absolute J_H values and (B) relative NHE activity, in cells subjected to a single acid pulse under conditions of normothermia (37°C, open bars and symbols) or moderate hypothermia (25°C, solid bars and symbols), in the presence of 0.01, 0.03, 0.1, 0.3, 1.0 or 3.0 μM cariporide ($n=8$ to 11 cells per group). *$P<0.05$ versus 37°C.

been assessed. In guinea-pig erythrocytes, lowering the temperature from 37 to 20°C has been shown to produce an increase in Na^+ influx that is sensitive to inhibition by the NHE inhibitor amiloride, which is indicative of a hypothermia-induced increase in NHE activity (Zhou & Willis, 1989). More recent work has shown that lowering the temperature from 37 to 27°C induces rapid swelling of rat glial cells in a manner that is inhibited by the amiloride analogue ethylisopropylamiloride, again suggesting increased NHE activity under conditions of moderate hypothermia (Plesnila *et al*., 2000). In contrast, the rate of swelling of rat lymphocytes following exposure to sodium propionate has been shown to be considerably slower at 22 and 27°C than at 37°C (and to be inhibited at each temperature by the NHE inhibitor FR168888), suggesting a hypothermia-induced decrease in NHE activity (Yamauchi *et al*., 1997). In the above studies (Plesnila *et al*., 2000; Yamauchi *et al*., 1997), NHE activity was surmized from the inhibitory effects of NHE inhibitors on the observed increase in cellular volume and direct measurements of NHE activity (i.e. the rate of NHE-mediated Na^+ influx or H^+ efflux) at known values of pH_i, which is the principal regulator of NHE activity (Wakabayashi *et al*., 1997), are scarce. In this context, Graber *et al*. (1992) have measured the rate of recovery of pH_i after the induction of an intracellular acid load in opossum kidney cells and shown this to be slower at 25°C than at 37°C. A similar observation has been reported in sheep cardiac Purkinje fibres, upon lowering of the ambient temperature from 37 to 22°C (Ellis & Macleod, 1985). Although these findings may indicate a reduction in NHE activity in the presence of moderate hypothermia, it is notable that, in both studies, the rate of recovery of pH_i was measured at a different level of intracellular acidosis under conditions of normothermia versus moderate hypothermia.

The present study is the first detailed characterization of the effects of moderate hypothermia on sarcolemmal NHE activity in adult mammalian ventricular myocytes, and demonstrates a significant inhibition of such activity upon lowering of the ambient temperature from 37 to 25°C. Notably, this inhibition is not absolute, such that at 25°C sarcolemmal NHE activity is retained at approximately 50–60% of that observed at 37°C. A recent preliminary report indicates that moderate hypothermia (27°C) may produce a similar degree of inhibition of sarcolemmal NHE activity in guinea-pig ventricular myocytes also (Ch'en & Vaughan-Jones, 2000). Interestingly, other evidence in the literature suggests that pathophysiologically significant sarcolemmal NHE activity may be retained even under conditions of severe hypothermia (<20°C). Thus, Na^+ has been shown to accumulate intracellularly during 6 h storage of embryonic chick cardiac myocytes at 10°C (Knerr & Lieberman, 1993) and 12 h storage of adult rat hearts at 4°C (Askenasy *et al*., 1996) in a manner that was significantly attenuated by the NHE inhibitor ethylisopropylamiloride.

In our experiments that employed two consecutive acid pulses, there was a marked increase in sarcolemmal NHE activity after the second acid pulse relative to the first, when both pulses occurred at 37°C (Figure 3). In contrast, no such increase in NHE activity was observed when the second pulse was at 25°C (Figure 3). In our previous studies that used similar 2-pulse protocols at 34°C (Avkiran & Yokoyama, 2000; Gunasegaram *et al*., 1999; Snabaitis *et al*., 2000; Yasutake *et al*., 1996; Yokoyama *et al*., 1998), there was only a small (<30%) increase in NHE activity after the second acid pulse (in the absence of any other intervention) and this increase was not statistically significant. It appears therefore that repeated episodes of intracellular acidosis can lead to increased sarcolemmal NHE activity, through a mechanism that is very sensitive to the ambient temperature. It would be of interest to determine the role of altered activity of NHE-regulatory signalling pathways (such as the protein kinase C and extracellular signal regulated kinase pathways (Moor & Fliegel, 1999; Snabaitis *et al*., 2000)) in such stimulation of sarcolemmal NHE activity by repeated episodes of acidosis. Regardless of the precise mechanisms underlying this interesting phenomenon, however, it is important to note that a similar reduction in sarcolemmal NHE activity by moderate hypothermia was evident also when cells were exposed to a single acid pulse (Figure 4). Therefore, it is likely that this negative effect of moderate hypothermia arose principally from the inhibition of sarcolemmal NHE activity *per se* rather than the attenuation of its stimulation by repeated episodes of intracellular acidosis.

The present work has also revealed that cariporide inhibits sarcolemmal NHE activity with comparable potency at 25

and 37°C, with an IC_{50} of 130–150 nM under each condition. Such an IC_{50} value is approximately 15 fold greater than that we have previously estimated for this drug in rat ventricular myocytes (Shipolini *et al*., 1997b). However, in our earlier study (Shipolini *et al*., 1997b), intracellular acidosis to activate the exchanger was induced in the absence of extracellular Na^+, which was reintroduced concomitantly with cariporide. In contrast, in the present study, intracellular acidosis was induced by the washout of NH_4Cl with Tyrode's solution, which contains Na^+ at a concentration of 137 mM. Extracellular Na^+ is known to antagonize competitively the binding of benzoylguanidine-based inhibitors such as cariporide to NHE (Baumgarth *et al*., 1998), which is likely to underlie the different IC_{50} values obtained in our studies. Indeed, in guinea-pig ventricular myocytes, the IC_{50} for HOE-694 (another benzoylguanidine-based NHE inhibitor that is closely related to cariporide structurally) has been estimated to be approximately 16 fold greater in the presence of an extracellular Na^+ concentration of 150 mM, relative to the value obtained in the virtual absence of extracellular Na^+ (Loh *et al*., 1996).

An interesting observation in the present study was the difference in basal pH_i under conditions of normothermia versus moderate hypothermia, such that this was 0.15–0.20 pH unit greater at 25°C than at 37°C. To our knowledge, this is the first report of this phenomenon in isolated ventricular myocytes, although similar effects of moderate hypothermia have been reported previously in sheep Purkinje fibres (pH_i increase of 0.21 (Ellis & Macleod, 1985) or 0.31 (Bright & Ellis, 1994) on reducing temperature from 35 to 21–22°C), isolated rat hearts (pH_i increase of 0.16 on reducing temperature from 36 to 20°C (Gruwel *et al*., 1998)) and sheep myocardium *in vivo* (pH_i increase of 0.19 on reducing temperature from 37 to 26°C (Swain *et al*., 1991)). Although the precise mechanism(s) underlying this increase in steady-state pH_i have not been determined, hypothermia-induced changes in the pK of intracellular buffers, such as the imidazole moiety of histidine, are likely to play an important role (Roos & Boron, 1981). In this context, it is notable that the pK of imidazole has been estimated to be 6.75 at 37°C but to increase to 7.30 at 25°C (Durand *et al*., 1998). On the basis that a low level of sarcolemmal NHE activity appears to be retained under steady-state conditions in ventricular myocytes (Leem *et al*., 1999), our data suggest that the inhibition of such activity may also contribute to the increase in basal pH_i during exposure to moderate hypothermia.

Previous data in sheep Purkinje fibres suggest that, under conditions of moderate hypothermia, the higher steady-state pH_i is associated with an attenuated level of intracellular acidification in response to the NH_4Cl pulse (Ellis & Macleod, 1985). Since pH_i is a critical determinant of NHE activity (Wakabayashi *et al*., 1997), we attempted to compensate for this and obtain comparable levels of intracellular acidosis in the normothermic control and moderate hypothermia groups, by extending the duration of the NH_4Cl pulse from 4 min at 37°C to 6 min at 25°C. This approach was only partially successful, however, in that the minimum pH_i achieved at 25°C remained approximately 0.10 pH unit higher than that at 37°C. This difference is unlikely to contribute to the lower NHE activity observed at 25°C (Figures 3 and 4), since J_H values were compared at identical pH_i values in the two groups. Nevertheless, in the cariporide study, where J_H was determined at the nadir of the acid pulse, a higher minimum pH_i value may have contributed to the lower NHE activity at 25°C. Indeed, in the presence of a non-inhibitory concentration of cariporide (0.01 μM), J_H at 25°C was only 45% of that at 37°C (Figure 6A). In contrast, when the comparison was made at identical values of pH_i in a similar protocol in the absence of cariporide (Figure 4), hypothermia-induced inhibition of sarcolemmal NHE activity was somewhat attenuated, with J_H values at 25°C measuring approximately 60% of those at 37°C.

The temperature-independence of the NHE-inhibitory potency of cariporide, at least within the temperature range that we have studied, suggests that this agent is likely to retain its cardioprotective efficacy under moderately hypothermic conditions. This is indeed borne out by our earlier work in isolated rat hearts (Shipolini *et al*., 1997a), which revealed that the use of cariporide as an additive to crystalloid cardioplegia improved the recovery of contractile function and reduced the leakage of creatine kinase following 120 min of global ischaemia at 28°C. This property is potentially important in relation to the application of cariporide for surgical myocardial protection and distinguishes this agent from other ion transport inhibitors, such as L-type calcium channel blockers. In this context, unlike cariporide (Shipolini *et al*., 1997a), verapamil (Hearse *et al*., 1984) and nifedipine (Fukunami & Hearse, 1985) have been shown to provide no significant cardioprotective benefit in isolated rat hearts when used as an additive to hyperkalaemic cardioplegia under conditions of moderate hypothermia, although both were effective at temperatures >30°C.

In conclusion, our work in isolated adult rat ventricular myocytes has shown that moderate hypothermia (25°C) produces a significant, but only partial, inhibition of sarcolemmal NHE activity. Furthermore, the NHE-inhibitory potency of cariporide is not affected by such a reduction in temperature. These findings may help provide a mechanistic basis for the previously demonstrated ability of cariporide to protect ischaemic myocardium under conditions of moderate hypothermia and suggest that this effect is likely to arise from the inhibition of retained NHE activity.

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