British Journal of Pharmacology (1996) 117, 831-838

Effects of trimetazidine on pH_i regulation in the rat isolated ventricular myocyte

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1 We have examined the effects of trimetazidine (TMZ) on intracellular pH (pH_i) regulation in rat isolated ventricular myocytes. pH_i was recorded ratiometrically by use of the pH-sensitive fluoroprobe, carboxy-SNARF-1 (carboxy-seminaphtorhodafluor).

2 Following an intracellular acid load (induced by 10 mM NH_4Cl removal), pH_i recovery in HEPES-buffered Tyrode solution was significantly slowed down upon application of 0.3 mM TMZ only when myocytes were pretreated for 5 h 30 min (slowing by $\sim$50%; $P<0.01$). This effect of TMZ on pH_i recovery was shown to be not only time- but also dose-dependent with a large, quickly reversible, effect obtained with 1 mM TMZ applied for 2-3 h (slowing by $\sim$64%; $P< 0.001$). This slowing of pH_i recovery was also associated with a decrease of the NH_4^+ removal-induced acidification.

3 Relationship between intracellular intrinsic buffering power (β_i) and pH_i was assessed in absence or presence of TMZ (0.3 mM or 1 mM). As expected, β_i increased roughly linearly with a decrease in pH_i in all cases. However, both concentrations of TMZ significantly increased β_i (by $\sim$55 and 65% at pH_i 7.1, respectively).

4 When Na^+/H^+ exchange was inhibited by dimethyl amiloride (DMA; 40 μM), trimetazidine (1 mM) did not change the H^+ flux estimated at pH_i 7.1 (0.31 ± 0.03 mequiv l^{-1} min^{-1}, $n=5$, control, *versus* 0.30 ± 0.025 mequiv l^{-1} min^{-1}, $n=5$, TMZ), ruling out any effect of TMZ on background acid loading.

5 Acid efflux carried by Na^+/H^+ exchange was significantly decreased only when myocytes were pretreated with 1 mM TMZ, for 2-3 h (J^e_H = 2.86 ± 0.38 mequiv l^{-1} min^{-1}, $n=26$, control, *versus* 1.66 ± 0.26 mequiv l^{-1} min^{-1}, $n=10$, TMZ, estimated at pH_i 7.1; $P<0.05$).

6 In conclusion, the present work demonstrates that, following an intracellular acid load in HEPES-buffered medium, trimetazidine slows down pH_i recovery in rat isolated ventricular myocytes, primarily through an increase of β_i. An effect on Na^+/H^+ exchange is also detected but only after long-term incubation of the myocytes with TMZ.

Keywords: Intracellular pH regulation; trimetazidine; cardiac ventricular myocytes; intracellular acidification; intrinsic buffering power; sodium-hydrogen exchange

Introduction

Changes in cellular cation homeostasis figure prominently in the pathogenesis of cellular damage during ischaemia and reperfusion (Tani, 1990; Vanheel *et al.*, 1990; Dennis *et al.*, 1991; Murphy *et al.*, 1991; Yan & Kléber, 1992). With respect to the functional alterations related to reperfusion of the ischaemic heart, it is now well recognized that re-activation of the Na^+/H^+ exchange, one of the major intracellular pH regulatory mechanisms, following ischaemia-induced acidosis, plays a key role in the development of such alterations (Khandoudi *et al.*, 1990). Several studies have indeed shown that inhibition of this exchange resulted in significant protection of the reperfused ischaemic myocardium (see Fliegel & Fröhlich, 1993, and Karmazyn & Moffat, 1993, for reviews).

Trimetazidine (TMZ, Vastarel), 1-(2,3,4-trimethoxybenzyl)-piperazine dihydrochloride, has been used for several years as an antianginal drug in clinical treatment of ischaemia. Previous work has suggested that TMZ may act at different levels of the sequence of events related to ischaemia. For example, in rat isolated heart preparations, TMZ improves the efficiency of oxygen consumption, protects against the fall in myocardial ATP content (Lavanchy *et al.*, 1987), and decreases the cellular accumulation of long chain acyl-carnitine (Feuvray, unpublished results). Of particular interest is the constant reduction, in the isolated heart, in ischaemic intracellular acidosis by TMZ (Lavanchy *et al.*, 1987; Aussedat *et al.*, 1993). This suggests that the mechanisms(s) of action of TMZ may be related to an effect of the drug on some pH_i regulatory mechanisms and/or to an interaction with metabolism. However, it was suggested that TMZ is ineffective on Na^+/H^+ exchange under basal conditions (Renaud, 1988). Excessive stimulation of glycolytic flux and lack of wash out during ischaemia leads to proton accumulation (Neely & Grotyohann, 1984). In this context, an alternative hypothesis for reducing ischaemia-induced intracellular acidosis may be through a reduction in glycolytic rate. However, measurements of glycolytic flux in isolated rat hearts perfused with TMZ have shown unchanged glucose utilization (Boucher *et al.*, 1994). Finally, another possible mechanism would be an increase of intracellular intrinsic H^+ buffering capacity, defined as the capacity of the cells to recruit several relatively rapid mechanisms that consume H^+ (Roos & Boron, 1981). Such an increase would indeed minimize the magnitude of the pH_i decrease.

In the present work, we have used the intracellular fluoroprobe, carboxy-SNARF-1, to investigate the effects of trimetazidine in rat isolated ventricular myocytes on: (i) pH_i recovery following an acid load in HEPES-buffered medium, (ii) the intracellular intrinsic H^+ buffering power (β_i), (iii) the activity of Na^+/H^+ exchange following an acid load, and (iv) the background acid loading (which is supposed to reflect glycolytic H^+ production; Vaughan-Jones & Wu, 1990a). Since it is now well recognized that Na^+/H^+ exchange plays a determinant role in the pathophysiology of ischaemia and reperfusion (see e.g. Karmazyn & Moffat, 1993 for review), we

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have chosen to use HEPES- rather than HCO_3^--buffered medium in order to inhibit the HCO_3^--dependent pH_i-regulating mechanisms, and hence to examine specifically the effects of trimetazidine on Na^+/H^+ exchange.

Methods

Animals

Male Wistar rats (250–300g) were used in this study. All procedures were in accordance with the regulations laid down by the Ministère de l'Agriculture et de la Forêt, France, for the care and use of laboratory animals.

Isolation of rat ventricular myocytes

Briefly, single ventricular myocytes were obtained from the hearts of rats (anaesthetized with pentothal, 50 mg kg^{-1} body weight, i.p.) using a combination of enzymatic (collagenase 0.28 mg ml^{-1}, Yakult, Japan; protease type XIV 0.05 mg ml^{-1}, Sigma Chemical Co., St. Louis, MO, U.S.A.). and mechanical dispersion. The composition of the basic solution and further details of the procedure have been described previously (Jourdon & Feuvray, 1993). Calcium-tolerant, rod-shaped ventricular myocytes were used on the day of isolation.

Experimental solutions

HEPES-buffered Tyrode solution contained (in mM): NaCl 140, KCl 5.4, $CaCl_2$ 1, $MgCl_2$ 1.2, glucose 11; HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid) 10, pH adjusted to 7.4 at 37°C with NaOH. In Na^+ -free Tyrode, NaCl was replaced with 140 mM N-methyl-D-glucamine and the pH adjusted to 7.4 at 37°C with HCl. When Tyrode solution with a pH of 6.8 was needed, PIPES buffer (piperazine-*N*,*N*'-bis[2-ethanesulphonic acid]) was used instead of HEPES buffer. Ammonium chloride (Sigma) was added directly to solutions shortly before use. Addition and then removal of NH_4Cl was used to induce an acid load in order to activate the pH_i-regulatory mechanisms (Boron & De Weer, 1976). Dimethyl amiloride (DMA, 40 μM; Sigma), an inhibitor of Na^+/H^+ exchange, was first dissolved in dimethylsulphoxide (DMSO) before addition to Tyrode solution (DMSO concentration <0.08%). Nigericin calibration solutions used in this study have been described elsewhere (Lagadic-Gossmann *et al.*, 1992).

Drug application protocol

Trimetazidine (TMZ), 1-(2,3,4-trimethoxybenzyl)-piperazine dihydrochloride, was obtained from the Institut de Recherches Internationales Servier, France. TMZ was added directly to the Tyrode solution and the final concentration was 0.3 mM or 1 mM (mol. wt. = 339.3). These concentrations were chosen since the effects of TMZ on pH_i in isolated hearts or cultured myocytes have been reported for concentrations of 0.3 and 0.6 mM (Lavanchy *et al.*, 1987; Renaud, 1988).

TMZ was applied to myocytes for 15 min (short-term treatment), 2 h, 3 h or 5 h 30 min (long-term treatment) prior to induction of the acid load (using the ammonium pre-pulse method; Boron & De Weer, 1976), and was present throughout the period of pH_i recording. When effects of short-term TMZ treatment were tested, control pH_i recovery and pH_i recovery in presence of TMZ were obtained from the same myocyte, whereas long-term treated cells were compared to control nontreated cells. In this study, we decided to pool results obtained from all control cells, since no time-dependent change in pH_i recovery was observed in this group (data not shown).

Measurement of pH_i

The pH_i of single isolated myocytes was monitored with the pH-sensitive fluorescent dye, carboxy-SNARF-1 (carboxyseminaphtorhodafluor; Molecular Probes, Eugene, U.S.A.) (Buckler & Vaughan-Jones, 1990). Cells were loaded with SNARF by incubating them in a 5 μM solution of the acetoxymethyl ester for 10 min at room temperature.

Carboxy-SNARF-1 fluorescence from individual cells was measured with an inverted microscope (Nikon Diaphot) converted to epifluorescence. SNARF-loaded cells were excited with light at 515 nm and the resulting fluorescence at 590 and 640 nm was measured with two photomultiplier tubes (Nikon, France). The signals were then digitized at 0.5 kHz (Cambridge Electronic Design, CED 1401 intelligent interface, U.K.) and stored for later analysis on the hard disk of a computer. The emission ratio 590/640 obtained from intracellular SNARF was calculated and converted to a linear pH scale using *in situ* calibration data obtained at the end of the experiment by the nigericin technique described elsewhere (Thomas *et al.*, 1979; Buckler & Vaughan-Jones, 1990). Finally, the calibrated pH_i signal was averaged over 0.5 s intervals.

Estimation of intracellular, intrinsic buffering power at different pH_i

The method used to estimate intracellular intrinsic buffering power (β_i) has been described previously (Vaughan-Jones & Wu, 1990b; Lagadic-Gossmann *et al.*, 1992). Briefly, a stepwise reduction of external NH_4Cl (from 20 mM) was applied to a selected myocyte. Each reduction in NH_4^+ resulted in the generation of intracellular H^+, due to dissociation of NH_4^+ into H^+ ions and NH_3, with subsequent efflux of NH_3. The resultant changes in pH_i were used to estimate β_i as follows:

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

where $[NH_4^+]_i = ([H^+]_i \times [NH_4^+]_O)/[H^+]_O$.
The experiments were carried out in the absence of extracellular Na^+ in order to prevent acid extrusion and barium (1 mM) to reduce NH_4^+ efflux through potassium channels (Vaughan-Jones & Wu, 1990b).

Calculation of sarcolemmal acid efflux

Details of the method for calculating acid efflux (J^e_H) during pH_i recovery in ventricular myocytes have been described previously (Lagadic-Gossmann *et al.*, 1992). Briefly, acid efflux was estimated using the following equation: $J^e_H = \beta_T . dpH_i/dt$, where β_T is the total intracellular buffering power and dpH_i/dt is the rate of pH_i recovery at any given pH_i. In HEPES-buffered medium, β_T equals the intrinsic buffering power β_i. In the present study, β_i at any given pH_i was calculated by use of one of the equations (1) to (3) in Results, depending on experimental conditions (these equations are empirical descriptions of the dependence of β_i upon pH_i under control conditions and in the presence of TMZ).

Statistics

All data are quoted as mean ± standard error of mean (s.e.mean) along with the number of observations, *n*. Student's *t* test or analysis of variance followed by Newman-Keuls test were used to test the effects of TMZ. Differences were considered significant at the level of $P<0.05$.

Results

Effects of trimetazidine on pH_i recovery following an acid load

Figure 1 shows a representative recording of intracellular pH in a single, isolated, ventricular myocyte of the rat obtained with the dual-emission fluoroprobe, carboxy-SNARF-1. The first part of the trace shows a control pH_i recovery from an

intracellular acid load, induced by addition and subsequent removal of 10 mM NH_4Cl (Boron & De Weer, 1976), in HEPES-buffered Tyrode solution. pH_i recovery following the second ammonium prepulse was obtained in the presence of 0.3 mM TMZ. Compared to control recovery, this did not appear to be significantly affected by the presence of the drug. Similar results were obtained in four cells. Thus, short-term (~15 min) application of 0.3 mM TMZ did not significantly change the rate of pH_i recovery following an acid load (Table 1), nor did it affect steady-state pH_i (7.15 ± 0.04, $n=4$, control, *versus* 7.15 ± 0.05, $n=4$, TMZ) and the pH_i value reached following the NH_4^+ removal (6.76 ± 0.03, $n=4$, control, *versus* 6.76 ± 0.03, $n=4$, TMZ).

In a previous paper, TMZ was shown to act in a time- and dose-dependent manner (Renaud, 1988). Therefore, ventricular myocytes were pretreated for longer periods of time (2 h, 3 h and 5 h 30 min) with the same concentration (0.3 mM) of TMZ prior the ammonium pre-pulse. Figure 2 clearly shows that the longer the pretreatment is, the slower is the pH_i recovery. The mean rates of pH_i recovery measured under control conditions and following the different durations of TMZ-pretreatments are given in Table 1. A significant slowing of pH_i recovery by TMZ (by ~50%) was obtained only in myocytes treated for 5 h 30 min. This effect was associated with a significant decrease of the amplitude of the NH_4^+ removal-induced acid load (Figure 3). Larger effects of TMZ were obtained when cells were pretreated for 2–3 h with a higher concentration (1 mM) of TMZ; thus, under these conditions, a significant reduction of the amplitude of the NH_4^+ removal-induced acid load was observed (ΔpH_i = 0.33 ± 0.02 pH_i unit, $n=11$ control cells, *versus* 0.21 ± 0.01 pH_i unit, $n=10$ TMZ pretreated cells; $P<0.05$), and the rate of pH_i recovery was decreased by ~64% (Table 2). Following washout (~10 min) after long-term incubation, the effects of TMZ were quickly reversible ($n=4$ similar experiments; data not shown).

As HEPES was used as extracellular buffer in all the experiments described above, Na^+/H^+ exchange was the main mechanism regulating pH_i following the acid load (Lagadic-Gossmann *et al.*, 1992). Therefore, taken altogether, the present results suggest that trimetazidine may slow the activity of Na^+/H^+ exchange. However, the observation that TMZ also induces a decrease of the acid load amplitude would rather be in favour of an increase in the intrinsic H^+ buffering power.

Effects of trimetazidine on intracellular intrinsic H^+ - buffering power, β_i

In the following set of experiments, we tested the hypothesis that the decrease of the acid load amplitude and the slowing of

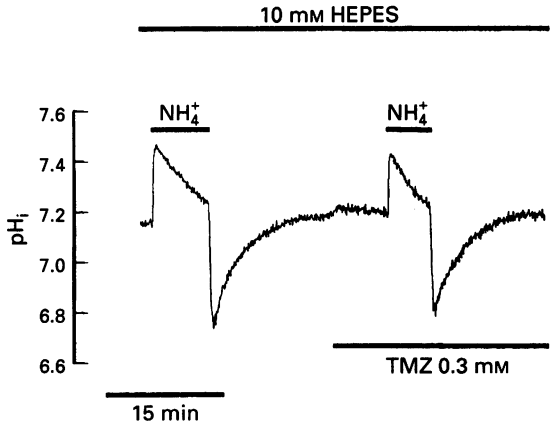

Figure 1 Effect of short-term application of trimetazidine (TMZ; 0.3 mM) on pH_i recovery from an intracellular acidification in HEPES-buffered Tyrode solution. Fluorimetric pH_i recording was by use of SNARF-1. The single myocyte was acid-loaded twice by the NH_4^+ prepulse method (10 mM NH_4Cl added and then removed from the superfusate; pH_O 7.4): first, in the absence (control recovery) and then in the presence of TMZ.

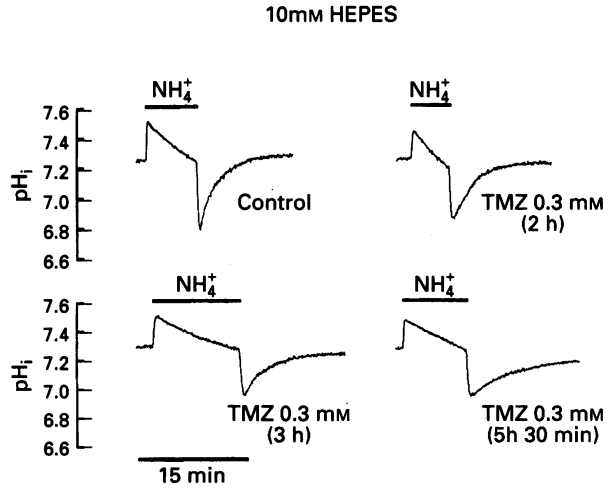

Figure 2 Effects of different pretreatment times with 0.3 mM TMZ on pH_i recovery. pH_i recordings were obtained from four different single myocytes. HEPES was used as extracellular buffer. Addition and removal of 10 mM NH_4Cl was used to induce an intracellular acid load.

Table 1 Effects of 0.3 mM trimetazidine on the rate of pH_i recovery following an NH_4^+ removal-induced acid load in ventricular myocytes

	dpH_i/dt (pH u min^{-1}) (–) 0.3 mM TMZ	dpH_i/dt (pH u min^{-1}) (+) 0.3 mM TMZ	pH_i
15 min	0.093 ± 0.01 ($n=4$)	0.074 ± 0.015 ($n=4$)	7.0
2 h	0.096 ± 0.008 ($n=26$)	0.077 ± 0.012 ($n=6$)	7.0
3 h	0.096 ± 0.008 ($n=26$)	0.068 ± 0.01 ($n=7$)	7.0
5 h 30 min	0.096 ± 0.008 ($n=26$)	0.048 ± 0.013** ($n=6$)	7.0

The pH_i value given in the last column indicates the value at which the rate of pH_i recovery was estimated. **$P<0.01$

pH_i recovery induced by TMZ were secondary to an increase of intracellular intrinsic buffering power (β_i). An increase of β_i would indeed slow pH_i recovery even in the absence of inhibition of acid extrusion through Na^+/H^+ exchange. We estimated β_i over the pH_i range 6.8–7.6 using the stepwise removal of external NH_4Cl (from 20 mM; see Methods). The procedure was conducted on three groups of myocytes: control myocytes (Figure 4, left trace), 0.3 mM TMZ-pretreated myocytes (for 5 h 30 min; Figure 4, right trace) and 1 mM TMZ-pretreated myocytes (for 2–3 h; not shown).

The resulting estimates of β_i at various values of pH_i are illustrated in Figure 5; it is clear from this figure that both TMZ-pretreatments significantly increased β_i whatever the value of pH_i. When fitted by a least square linear regression, these estimates of β_i could be described by the following equations: (1) $\beta_i = 127.5 - 13.3 \times pH_i$ for the control group ($r = 0.85$; $n = 22$ cells), (2) $\beta_i = 178.5 - 17.9 \times pH_i$ for the 0.3 mM TMZ-pretreated group ($r = 0.85$; $n = 10$ cells), and (3) $\beta_i = 126.7 - 10.2 \times pH_i$ for the 1 mM TMZ-pretreated group ($r = 0.75$; $n = 10$ cells). These equations indicate that, at pH_i 7.1, β_i will be 33.1 mM (control), 51.4 mM (0.3 mM TMZ) and 54.3 mM (1 mM TMZ).

Therefore, treatment of rat ventricular myocytes with either 0.3 mM (for 5 h 30 min) or 1 mM (for 2–3 h) trimetazidine resulted in a significant and similar rise of β_i, responsible for at least part of the slowing of pH_i recovery.

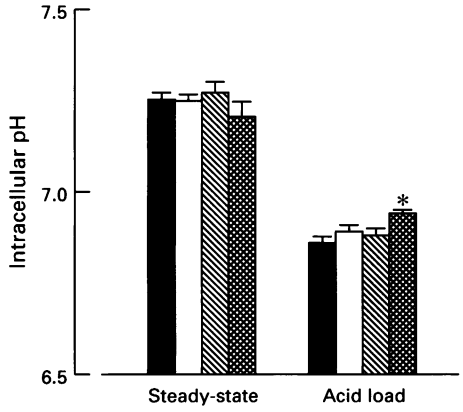

Figure 3 Effects of 0.3 mM TMZ on pH_i recorded under steady-state conditions and at the peak of the acid load following NH_4^+ removal. It should be pointed out that, in all experiments, NH_4^+ was removed from Tyrode solution at roughly the same pH_i. Each column gives the mean value ± s.e.mean of 26, 6, 7 and 6 experiments for control (solid columns), TMZ 2 h (open columns), TMZ 3 h (hatched columns) and TMZ 5 h 30 min (cross-hatched columns), respectively. *$P < 0.05$ *versus* control.

Table 2 Effects of 1 mM trimetazidine on the rate of pH_i recovery following an NH_4^+ removal-induced acid load in ventricular myocytes

	dpH_i/dt (pH u min^{-1})	pH_i
Control	0.086 ± 0.011 ($n = 26$)	7.1
+ 1 mM TMZ (2 h–3 h)	0.031 ± 0.005*** ($n = 10$)	7.1

The pH_i value given in the last column indicates the value at which the rate of pH_i recovery was estimated. ***$P < 0.001$

Effects of trimetazidine on background acid loading

Slowing of pH_i recovery upon TMZ treatment could have also resulted from an increase in background acid loading (background loading resulting from cell metabolism and/or from proton influx via HCO_3^--dependent mechanisms; it has been suggested that such mechanisms are active even in HEPES-buffered media; see e.g. Wu *et al.*, 1994).

Figure 6 shows experiments where Na^+/H^+ exchange was inhibited by addition of 40 μM dimethyl-amiloride (DMA), which led to a slow acid loading of the myocytes. From these experiments, it appears that the rate of this acid loading was reduced by pretreatment with 1 mM TMZ (for 2–3 h) (at pH_i 7.1, $dpH_i/dt = -0.009 \pm 0.001$ pH_i unit min^{-1}, $n = 5$ control cells, *versus* -0.0055 ± 0.0005 pH_i unit min^{-1}, $n = 5$ TMZ-pretreated cells; $P < 0.05$). However, when calculating H^+ flux using the β_i values estimated above, no significant difference could be found between control conditions (0.31 ± 0.03 mequiv l^{-1} min^{-1}, $n = 5$, at pH_i 7.1) and following TMZ pretreatment (0.30 ± 0.025 mequiv l^{-1} min^{-1}, $n = 5$, at pH_i 7.1).

We therefore conclude that a change in background acid

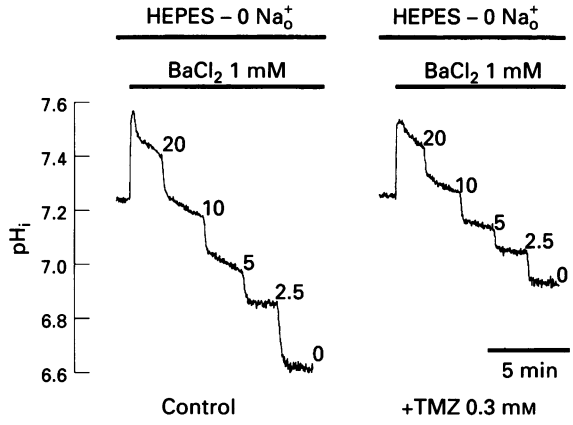

Figure 4 Representative experiments carried out in order to test the effect of trimetazidine (TMZ) on pH_i dependence of intracellular intrinsic buffering power (β_i) in the rat isolated ventricular myocyte. The cells were superfused with Na_O-free HEPES-buffered Tyrode solution containing $BaCl_2$. External NH_4Cl concentration was reduced as indicated by the figures (in mM) next to the traces. For the treated myocyte (right trace), TMZ (0.3 mM) was added 5 h 30 min prior to the experiment.

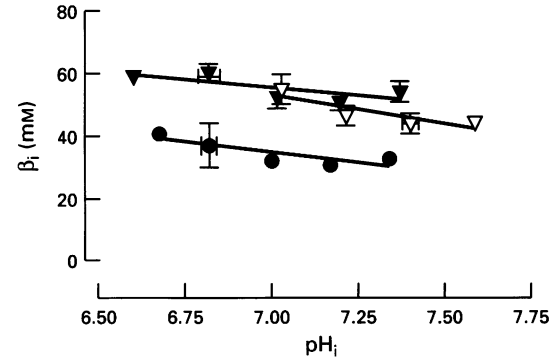

Figure 5 Graph showing the average effects of trimetazidine on pH_i-dependence of intracellular intrinsic buffering power (β_i). Cells were pretreated either with 0.3 mM TMZ for 5 h 30 min ($n = 10$; ▽) or with 1 mM TMZ for 2–3 h ($n = 10$; ▼). $n = 22$ cells for control conditions (●). The mean ± standard error of β_i is plotted *versus* the mean ± s.e. of pH_i (i.e. the mid-point of each stepwise acid load). Pooled data have been averaged over the following range of pH_i: 6.5–6.7; 6.7–6.9; 6.9–7.1; 7.1–7.3; 7.3–7.5; 7.5–7.7. In each group, data were fitted by a linear least-squares regression analysis.

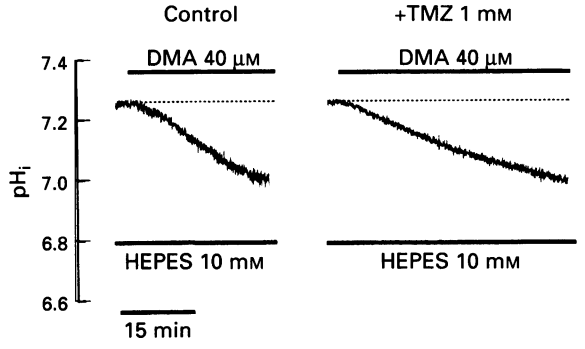

Figure 6 Effect of trimetazidine on background acid loading. Dimethyl amiloride (DMA; 40 μM) was added to the superfusate in order to inhibit acid extrusion. pH_i recordings show the time course of pH_i decrease upon DMA application under control conditions (left trace) and following TMZ (1 mM; applied for 3 h) pretreatment (right trace). Dotted lines: steady-state pH_i values.

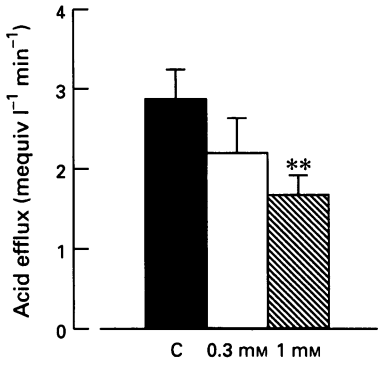

Figure 7 Histogram illustrating the effect of both concentrations of trimetazidine (TMZ) on the mean acid efflux (J^e_H) estimated at pH_i 7.1 in HEPES-buffered Tyrode solution, i.e. mainly on the efflux carried by Na^+/H^+ exchange. Solid column: control cells (C; $n=26$); open column: cells pretreated with 0.3 mM TMZ for 5 h 30 min ($n=6$); hatched column: cells pretreated with 1 mM TMZ for 2–3 h ($n=10$). $^{**}P<0.01$, TMZ *versus* control.

loading in our quiescent isolated myocytes cannot account for the observed effects of trimetazidine upon pH_i recovery from an intracellular acid load.

Effects of trimetazidine on the acid efflux carried by Na^+/H^+ exchange

Figure 7 shows the effects of 0.3 mM TMZ-pretreatment and of 1 mM TMZ-pretreatment on the acid efflux (J^e_H; see Methods for determination) estimated in HEPES-buffered Tyrode solution. Under these conditions, the acid efflux is mostly carried by Na^+/H^+ exchange. Whereas 0.3 mM TMZ, even after long-term pretreatment of myocytes, exerted a slight, but not significant, effect on Na^+/H^+ exchange (acid efflux decreased by ~23%), incubating cells with 1 mM TMZ for 2–3 h resulted in a significant reduction (by ~42%) of the acid efflux carried by this exchange.

Effects of trimetazidine on pH_i recovery at pH_o 6.8

Cardiac ischaemia is associated with an intracellular acidification which rapidly leads to an extracellular acidification (Yan & Kléber, 1992). The following experiments were carried out in order to test the effects of superfusing myocytes with an acidic medium (pH_o 6.8) on the action of trimetazidine upon pH_i recovery. Such experiments are illustrated in Figure 8. As previously demonstrated (Vaughan-Jones & Wu, 1990a), pH_i recovery was slowed down at pH_o 6.8 as compared to that obtained at pH_o 7.4. Figure 8a also shows that pretreatment of myocytes with 1 mM TMZ (for 2–3 h; superimposed trace) induced a further slowing of pH_i recovery under such conditions. Therefore, the effect of TMZ was maintained at pH_o 6.8, though less important than that obtained at pH_o 7.4 (Figure 8b).

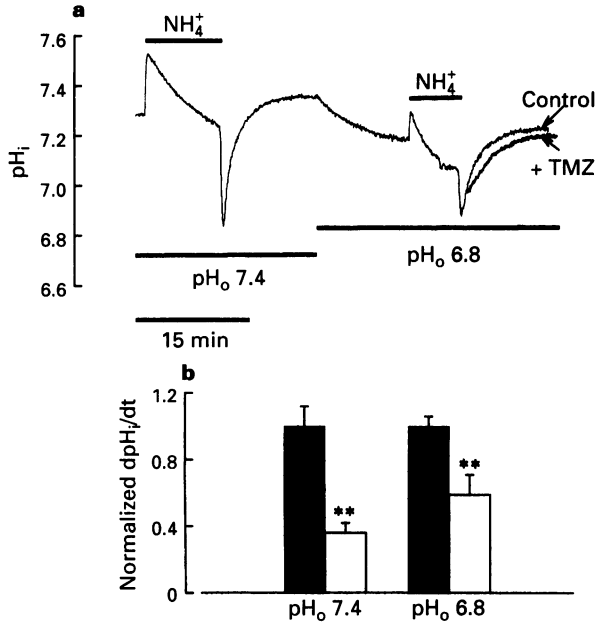

Figure 8 Effect of trimetazidine (1 mM) on pH_i recovery at extracellular acidic pH (6.8). Panel (a) shows a typical experiment in which pH_i recovery from an acid load was recorded at pH_o 7.4 (first part) and pH_o 6.8 (second part). The pH_i recovery obtained at pH_o 6.8 from a different myocyte previously pretreated with TMZ (1 mM for 3 h) was superimposed on the control trace (right part) in order to outline the effect of TMZ. Histogram in (b) illustrates the effect of TMZ on the normalized (to control) rate of pH_i recovery estimated at pH_o 7.4 ($n=26$ control and 10 TMZ-pretreated cells; pH_i 7.1) and at pH_o 6.8 ($n=6$ control and 6 TMZ-pretreated cells; pH_i 7.0). Solid columns: control cells; open columns: TMZ-pretreated cells. $^{**}P<0.01$.

Discussion

The present work demonstrates that pretreatment (up to 5 h 30 min) of rat isolated ventricular myocytes with trimetazidine (0.3 mM or 1 mM) significantly reduces the amplitude of the NH_4^+ -induced acid load and slows the subsequent pH_i recovery. Superfusing of the cells with an acidic medium (pH_o 6.8) did not suppress such an effect, although, under these conditions, the effect was less marked. The fact that the action of TMZ on pH_i recovery appears to be dose- and time-dependent seems to indicate that TMZ needs to accumulate inside the myocytes in order to exert its effects on pH_i. Moreover, our data suggest that this accumulation may be pH_o-dependent since a less pronounced effect of TMZ was observed at pH_o 6.8.

An interesting observation is that the slowing of pH_i recovery by TMZ is associated with a significant increase of intracellular intrinsic buffering power, β_i. An effect of TMZ on the acid efflux carried by Na^+/H^+ exchange was also observed, but only when cells were pretreated for 2–3 h with the highest concentration (1 mM) of TMZ. Therefore, it appears that the cellular mechanisms involved in the action of trimetazidine on cardiac pH_i regulation are dose- and time-dependent, with the primary effect being through the intracellular intrinsic buffering power, β_i.

Recently, a similar effect on β_i has been reported in cardiac

Purkinje fibres following inhibition of oxidative metabolism by cyanide (Wu & Vaughan-Jones, 1994). In this latter work, the rise in inorganic phosphate (P_i) resulting from hydrolysis of phosphocreatine (PC) and ATP upon cyanide application was proposed to be responsible for the increase of β_i. In this context, we may similarly suppose that the present effect of TMZ on β_i would involve changes in cellular metabolism, that may include an increase of P_i level. However, it has been shown that, during both ischaemia and long-term preservation of heart, TMZ protected myocardial ATP content rather than accelerated its degradation (Lavanchy *et al.*, 1987; Aussedat *et al.*, 1993). Furthermore, a decrease of P_i level has even been observed under normoxic conditions at a TMZ concentration of 0.6 mM (that is in the same range as that used in this study), this decrease becoming highly manifest during reperfusion of ischaemic heart (Lavanchy *et al.*, 1987). Therefore, the effect of TMZ on myocardial β_i is unlikely to be due to an increase in P_i level. Alternatively, an increase in organellar H^+ buffering upon application of TMZ might underlie the changes in β_i observed in this study. In this regard, it has been suggested that TMZ treatment would induce a more efficient use of oxygen by cardiac mitochondria (Lavanchy *et al.*, 1987; Aussedat *et al.*, 1993), which might stimulate mitochondrial consumption of protons through ATP production. Finally, another possible explanation is that TMZ, as a weak base, would play a direct role in physicochemical buffering of the cell. This type of buffering, probably the single most important buffer system, is a property of weak acids and bases whereby these compounds minimize shifts in pH by reacting directly with exogeneous H^+ (Roos & Boron, 1981). In summary, our data demonstrate that TMZ treatment of cardiac cells enhances β_i, thus facilitating the rapid neutralization of intracellular protons, that is, the short-term pH_i homeostasis (Roos & Boron, 1981). With respect to the long-term pH_i homeostasis, that relies on the membrane acid-extrusion mechanisms, we have shown that TMZ decreased the activity of Na^+/H^+ exchange, but only when the drug was used at high concentration.

This slowing of Na^+/H^+ exchange might occur in several ways. For example, TMZ treatment might elevate Na^+_i, possibly by changing the activity of Na^+/K^+-ATPase. Raising Na^+_i in lymphocytes can slow Na^+/H^+ exchange (Grinstein *et al.*, 1984) although this effect has yet to be demonstrated in cardiac cells. It has been shown that TMZ (15 μM and 150 μM) inhibited Na^+/K^+-ATPase in guinea-pig ventricular muscle (Hisatome *et al.*, 1991). However, this negative effect is still controversial since TMZ (300 μM), even after a 12 h-pretreatment period, has been found to be ineffective on the Na^+/K^+-ATPase in chick embryonic cardiac cells (Renaud, 1988). Therefore, elevation of Na^+_i upon TMZ-treatment in rat ventricular cells remains to be demonstrated. It has been shown that the activity of Na^+/H^+ exchange is controlled by different intracellular messengers such as cyclic AMP_i, Ca^{2+}_i or products of phosphoinositide metabolism such as diacylglycerol (DAG), a membrane regulator of protein kinase C (PKC) (see e.g. Counillon & Pouysségur, 1995; Noël & Pouysségur, 1995, for reviews). A derangement of intracellular signalling systems upon TMZ treatment may therefore be an additional explanation for the slowing of Na^+/H^+ exchange. In this respect, it is worthwhile to point out that elevation in cyclic AMP_i level has been shown to inhibit the activity of cardiac Na^+/H^+ exchange (Lagadic-Gossmann *et al.*, 1993; Wu & Vaughan-Jones, 1994). However, in human platelets, TMZ has been shown to exert no effect on basal cyclic AMP levels and even to reduce this level slightly in the presence of a phosphodiesterase inhibitor (Devynck *et al.*, 1993). Finally, we may suppose an action of TMZ on membrane fluidity, known to affect the activity of Na^+/H^+ exchange (Dudeja *et al.*, 1987). Up to now, no data are available concerning the effect of TMZ on the membrane fluidity of cardiac cells; nevertheless, in human platelets, TMZ has been suggested to decrease selectively the fluidity of the outer part of the plasma membrane (Devynck *et al.*, 1993), which consequently might decrease the activity of Na^+/H^+ exchange (Dudeja *et al.*, 1987). Since HEPES was used as extracellular buffer in this study, further work is still required to evaluate the action of TMZ on the HCO_3^--dependent mechanisms, especially on the Na^+-HCO_3^- co-transport, known to be activated in parallel with Na^+/H^+ exchange (Lagadic-Gossmann *et al.*, 1992). Should any of the cellular modifications described above occur upon TMZ-treatment, one could then also expect effects on the co-transport since its activity is dependent on Na^+_i (Lagadic-Gossmann *et al.*, 1992) and is modulated by intracellular messengers (Lagadic-Gossmann *et al.*, 1993).

It has become clear that most pathophysiological processes in myocardial ischaemia and reperfusion are connected to a derangement of cellular ion homeostasis, with Na^+/H^+ exchange and Na^+/Ca^{2+} exchange as determinant factors (see e.g. Karmazyn & Moffat, 1993, and Pierce & Czubryt, 1995, for reviews).

Several works have reported that TMZ significantly decreased the amplitude of the ischaemia-related acidification in isolated hearts (Lavanchy *et al.*, 1987; Aussedat *et al.*, 1993), thereby minimizing the availability of internal protons. Under these conditions, the activity of Na^+/H^+ exchange, and consequently Na^+/Ca^{2+} exchange, would then be less stimulated. In this respect, it is important to note that, under acid-load conditions, TMZ has been shown to limit accumulation of Na^+ and Ca^{2+} inside cardiac cells (Renaud, 1988). Until now, two main hypotheses have been put forward to explain the TMZ-induced reduction of the acidosis in ischaemia: (1) a decrease in metabolic H^+ production (through a decrease of the glycolytic flux), or (2) the maintenance of some hydrogen ion transport. With respect to the former hypothesis, it has been shown recently that TMZ did not modify myocardial exogenous glucose utilization during either low-flow ischaemia or postischaemic reperfusion (Boucher *et al.*, 1994). Moreover, the present results show that under normoxic conditions, the background acid loading, mainly due to glycolytic H^+ production in quiescent rat isolated ventricular myocytes (Le Prigent & Lagadic-Gossmann, unpublished results), is unaffected by TMZ treatment. Therefore, a decrease in metabolic H^+ production may not be the major cause of the TMZ-induced reduction of the acidosis observed in ischaemia. Concerning the maintenance of pH_i-regulating mechanisms in ischaemia, this appears to be improbable since TMZ, when used at high concentration (the only situation where an effect was observed), *inhibited*, rather than stimulated, Na^+/H^+ exchange. However, at present, one cannot eliminate any contribution of the Na^+-HCO_3^- co-transport in the TMZ-induced reduction of acidosis observed in isolated hearts, perfused with HCO_3^- as extracellular buffer (Lavanchy *et al.*, 1987; Aussedat *et al.*, 1993). In a previous report, Tani and coworkers (1993) have demonstrated that enhancement of the H^+ buffering capacity of the perfusate (by administration of HEPES) before ischaemia attenuated myocardial stunning while improving the Na^+ imbalance and Ca^{2+} overload. In this context, an alternative explanation, that we have considered in this work, was that TMZ, by accumulating inside the cells, modified the *intracellular* H^+ buffering capacity of cardiac cells. This is the first study to demonstrate that TMZ significantly increases β_i (by 55–65%) in rat isolated ventricular myocytes. This effect is likely to participate in the TMZ-induced reduction of the acidosis observed in ischaemia in the presence of bicarbonate (Lavanchy *et al.*, 1987), since the amplitude of the NH_4^+-induced acid load was found to be significantly reduced by TMZ treatment (present study), and β_i is known to be independent of the extracellular buffer used (Roos & Boron, 1981). Therefore, the beneficial effect of TMZ during the sequence ischaemia-reperfusion appears to act partly through an effect on β_i, which would represent a novel type of action mechanism for anti-ischaemic drugs.

Besides the effect of TMZ on β_i, we have also found an effect on Na^+/H^+ exchange. Under these conditions, the slowing of Na^+/H^+ exchange upon TMZ treatment would then further reduce Na^+ influx and consequently Ca^{2+} overload during ischaemia and especially on reperfusion. Con-

cerning Na^+/Ca^{2+} exchange, its activity is not affected by TMZ (Renaud, 1988). In summary, TMZ treatment, primarily by increasing cardiac β_i and eventually and additionally by slowing the activity of Na^+/H^+ exchange, would result in a net reduction of the Ca^{2+} overload during reperfusion of an ischaemic heart.

A major question concerns the therapeutic relevance of the results reported here, since we used TMZ concentrations higher than those found to limit the consequences of the sequence ischaemia-reperfusion *in vivo* and *in vitro* (in the range 0.1 – 1 μM). We must however emphasize that TMZ efficiency appears to increase with the incubation time (Renaud, 1988 and present study) and that the sensitivity of the target cells is described as being higher under pathological conditions such as experimental acidosis or ischaemia (Renaud, 1988). This also agrees with the fact that TMZ administered chronically is more efficient at lower concentrations than when given acutely (Lavanchy *et al.*, 1987; Fabiani *et al.*, 1992; Kober *et al.*, 1992). Further studies are now needed to investigate whether the intracellular H^+ buffering capacity of cardiac cells may be modified in animals chronically treated with TMZ.

In conclusion, we have demonstrated that, following an acid load in HEPES-buffered media, trimetazidine reduces the amplitude of an ammonium-induced acid load and slows down pH_i recovery in rat isolated ventricular myocytes. This occurs primarily through an increase of the intracellular H^+ buffering capacity of the cells. Such an increase of cardiac intracellular H^+ buffering capacity may well limit the availability of internal protons during ischaemia and upon reperfusion of the ischaemic heart, and would thus represent a novel type of action mechanisms for anti-ischaemic drugs.

This work was supported by the Institute de Recherches Internationales Servier, France. We wish to thank A. Lucien of I.R.I. Servier for helpful suggestions.

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(Received July 14, 1995
Revised October 2, 1995
Accepted November 1, 1995)