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Effect of Hoe 694, a novel Na^+-H^+ exchange inhibitor, on intracellular pH regulation in the guinea-pig ventricular myocyte

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1 Hoe 694 (3-methylsulphonyl-4-piperidinobenzoyl, guanidine hydrochloride) is a Na^+/H^+ exchange (NHE) inhibitor exhibiting cardioprotective properties during ischaemia and reperfusion in animal hearts. We have (i) tested the selectivity of Hoe 694 for NHE over other pH_i-regulating mechanisms in the myocardium, and (ii) tested if the functionally important NHE isoform contributing to intracellular pH regulation in heart is NHE-1, as suggested from molecular biology studies of this protein.

2 pH_i was recorded by fluorescence microscopy with carboxy SNARF-1, AM-loaded into single ventricular myocytes of guinea-pig.

3 In nominally HCO_3^--free media, recovery of pH_i from an intracellular acid load is mediated by NHE, and was inhibited by Hoe 694, amiloride (an NHE inhibitor) or dimethyl amiloride (DMA, a high affinity NHE inhibitor) with potency values of 2.05, 87.3 and 1.96 μM respectively, giving the potency series: Hoe 694 $\simeq$ DMA $>>$ amiloride. This potency series, and the potency values (corrected for drug competition with extracellular Na^+) match those determined previously for cloned NHE-1 expressed in mutant fibroblasts. In the absence of extracellular Na^+ (to inhibit NHE), Hoe 694 had no effect on pH_i.

4 In 5% CO_2/HCO_3^--buffered solution containing DMA, pH_i recovery from acidosis is mediated by Na^+-HCO_3^- symport and was unaffected by Hoe 694. The drug also had no effect on pH_i recovery from an alkali-load, a process largely mediated by Cl^--HCO_3^- exchange. Finally, the fall of pH_i upon adding extracellular Na-lactate is assisted by H^+-lactate symport, and this too was unaffected by Hoe 694.

5 We conclude (i) Hoe 694 has no detectable inhibitory potency for pH-regulating carriers in heart other than NHE. (ii) native NHE functioning during pH_i-regulation in the cardiomyocyte is the NHE-1 isoform. These data strengthen the case for NHE-1 being the receptor for mediating the cardioprotective effects of Hoe 694.

Keywords: Hoe 694; intracellular pH; heart; cardiac; myocyte; SNARF; Na^+/H^+ exchange; Na^+/HCO_3^- symport; Cl^-/HCO_3^- exchange; lactate/H^+ symport

Introduction

Na^+-H^+ exchange (NHE) is important for intracellular pH (pH_i) control in heart, being one element of a multi-carrier acid-equivalent transport system in the sarcolemma of the cardiac cell (Lagadic-Gossmann *et al.*, 1992). NHE proteins comprise a family of at least four NHE isoforms (NHE 1–4), each one expressed by a separate gene. The mRNA for NHE 1 has been identified in cardiac cells (Fliegal *et al.*, 1991; Orlowski *et al.*, 1992), and the cardiac NHE 1 protein has been cloned and sequenced (Fliegel *et al.*, 1991). As yet, however, there is no clear pharmacological evidence for the functional expression of native NHE 1 in cardiac cells. Evidence for this has awaited the development of highly selective inhibitors of the NHE 1 isoform. Although truly selective compounds have still to be produced, an important step in this direction was made recently with the discovery of a new class of NHE inhibitor (Scholz *et al.*, 1993). Compounds of this class are benzoyl guanidine derivatives, a potent example of which is Hoe 694 (3-methylsulphonyl-4-piperidinobenzoyl, guanidine hydrochloride). This compound shows a selectivity for cloned and expressed NHE 1 that is two or more orders of magnitude higher than for the isoforms NHE 2 and 3 (Counillon *et al.*, 1993), and it has recently been shown to inhibit NHE in perfused rat and rabbit hearts *in vitro* (Scholz *et al.*, 1993; Hendrikx *et al.*, 1994). The more classical NHE inhibitors (amiloride and its analogues, such as dimethyl amiloride [DMA]) which are also guanidine compounds, show much lower selectivity among the cloned and expressed NHE isoforms. Counillon *et al.* (1993) have suggested recently that the *differential inhibitory effects* of Hoe 694, amiloride and DMA might provide a pharmacological means of identifying particular NHE-isoforms in a given cell type. In the present work, we have tested this idea by using the above three NHE inhibitors to produce a pharmacological profile of native, cardiac NHE under conditions where it is responsible for intracellular pH (pH_i) regulation in myocytes isolated enzymatically from guinea-pig ventricle. The pH_i was measured with the intracellular fluoroprobe carboxy SNARF-1. Our results indicate that the native isoform contributing to acid extrusion and hence to pH_i regulation in heart must be NHE 1.

Hoe 694 has recently been found to be remarkably effective at suppressing post-ischaemic, reperfusion arrhythmias and cellular, reperfusion damage in animal models (Scholz *et al.*, 1993). This cardioprotective property of Hoe 694 is attracting considerable interest, and is attributed to the inhibitory action of the drug on NHE 1 in cardiac cells. One problem with this interpretation is that very little is known about the selectivity of Hoe 694 for NHE over other ion transport proteins in heart. In particular, it is not known if Hoe 694 affects other acid-equivalent carriers in the sarcolemma. We have therefore screened all the known types of sarcolemmal, acid-equivalent carrier in the cardiomyocyte for sensitivity to Hoe 694. We find no sensitivity of any acid equivalent carrier other than NHE. This strengthens the hypothesis that the cardioprotective properties of Hoe 694 are dependent on its ability to target and inhibit cardiac NHE-1.

A preliminary account of some of these findings has been published (Loh & Vaughan-Jones, 1995).

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Methods

Isolation of guinea-pig ventricular myocytes

Ventricular myocytes were isolated from the hearts of albino guinea-pigs, (350–450 g, killed by cervical dislocation) by use of a combination of enzymatic (collagenase 0.5 mg ml^{-1}, Worthington Chemical Co., Type I and protease 0.1 mg ml^{-1}, sigma Type XIV) and mechanical dispersion (Powell *et al.*, 1980; Levi *et al.*, 1990). The composition of the basic solution used for the isolation was (mM): NaCl 128, KCl, 2.5, $MgCl_2$ 1.2, KH_2PO_4 1.2, HEPES 10; taurine 20 glucose 11; titrated to pH 7.25 at 37°C; to which calcium, EGTA or enzyme was added as required. After dissociation, the cells were suspended in Dulbecco's modified Eagle's medium (DMEM, Sigma) at room temperature until use (Lagadic-Gossmann *et al.*, 1992). Only myocytes displaying a rod shape and calcium tolerance were used in the present study.

Solutions

Standard HEPES-buffered Tyrode solution (air equilibrated) contained (mM): NaCl 140, KCl 4.5, $MgCl_2$ 1, $CaCl_2$ 2.5, glucose 11, HEPES 20, pH adjusted to 7.4 with 4 N NaOH. Unless otherwise stated, pH adjustments of all HEPES-buffered solutions (including those where ionic-substitutions were made, see below) were performed at 37°C. Standard bicarbonate-buffered Tyrode solution (equilibrated with 5%CO_2/23 mM HCO_3^-) was the same as above, except that the sodium chloride concentration was reduced to 117 mM and 23 mM $NaHCO_3$ was added instead of the HEPES (pH 7.40 at 37°C).

Ion-substituted solutions: in Na^+-free, HEPES-buffered Tyrode solution, NaCl was replaced with 140 mM N-methyl-D-glucamine (NMDG), and the pH adjusted to 7.4 with HCl. Cl^--free, CO_2/HCO_3^--buffered Tyrode solution contained (mM): sodium glucuronate 117, potassium gluconate 4.5, calcium gluconate 12, $NaHCO_3$ 23, $MgSO_4$ 1; glucose 11. In acetate-containing bicarbonate-buffered Tyrode solution, 40 mM sodium acetate was added to the standard bicarbonate-buffered Tyrode solution and an equivalent concentration of NaCl was omitted. The solutions used while investigating the lactate transport carrier (e.g. Figure 6) contained (mM): sodium citrate 100, HEPES 20, pH adjusted (NaOH) to 7.40 at 25°C. When 10 mM ammonium chloride was used, it was added directly as solid to solution without osmotic compensation. Amiloride, 5-N-dimethyl amiloride (DMA), 4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid (DIDS) and Hoe 694 (3-methylsulphonyl-4-piperidinobenzoyl, guanidine hydrochloride) were added, as solids, to solutions shortly before use.

Nigericin calibration solutions contained (mM): KCl 140; $MgCl_2$ 1, 10 μM nigericin; buffered with one of the following organic buffers: 20 mM 2-(N-morpholino) ethanesulphonic acid (MES, pH 5.5), 20 mM HEPES (pH 7.5) or 20 mM 3-(cyclohexylamino)-2-hydroxy-1-propane-sulphonic acid (CAPSO, pH 9.5), and were adjusted (37°C) to the correct pH with 4N NaOH.

Hoe 694 was kindly provided by Hoechst Akitengesellschaft (Germany). All other chemicals were from Sigma (UK) and Merck (UK).

Measurement of intracellular pH

The pH_i of single isolated myocytes was measured with the pH-sensitive fluorescent dye carboxy-SNARF-1 (Buckler & Vaughan-Jones, 1990). Cells were loaded with SNARF by incubating them in a 5–10 μM solution of the acetoxy-methyl ester for 8–10 min at room temperature. Carboxy-SNARF-1 fluorescence from individual cells was measured with an inverted microscope (Nikon Diaphot) converted for epi-fluorescence. Autofluorescence from these cells was found to be negligible. A selected cell was excited with light at 540 ± 12 nm. The resulting fluorescent-emission was measured simultaneously at 590 ± 5 nm and 640 ± 12 nm, with two photomultiplier tubes linked to a current-voltage converter. The signals were then digitized at 0.5 kHz (Cambridge Electronic Design, CED 1401 intelligent interface) and stored on the hard disk of a microcomputer for later analysis. The emission ratio 590 nm/640 nm, was calculated and converted to a linear pH scale using *in situ* calibration data obtained by use of the nigericin technique (Thomas *et al.*, 1979), as described previously (Buckler & Vaughan-Jones, 1990; Lagadic-Gossmann *et al.*, 1992). Briefly, this consisted of exposing a SNARF-loaded cell to the three nigericin calibration solutions (listed above). This clamps pH_i to the value of pH_0 in the calibration solution (Thomas *et al.*, 1979). The intracellular emission ratio is measured at pH 5.5 (R_{max}), 9.5 (R_{min}) and at 7.5 ($R_{7.5}$), as well as the maximum/minimum emission intensity (pH 9.5/pH 5.5) recorded at 640 nm ($F640_{max/min}$). These terms were then used to convert intracellular fluorescence ratio (R) to pH_i, using the following equation: $pH = pK_a - \log(R - R_{min})/(R_{max} - R) - \log F640_{max/min}$, where pK_a is the pK of intracellular SNARF (typically about 7.6; pK_a can be estimated from the above equation for the individual cases where both R and pH_i are known, i.e. during application of one of the nigericin solutions). Finally, the calibrated pH_i signal was averaged over 0.5 s intervals.

Nigericin calibration of *in situ* cell fluorescence was not performed after every experiment. Typically it was performed on at least 9 cells (minimum of 2 animals). Mean values of R_{max}, R_{min} and $R_{7.5}$ were then determined and were used, in subsequent experiments, as default values in the calculation of pH_i from the SNARF fluorescence ratio signal. In order to guard against inaccuracies in pH_i calibration developing over periods covering several experiments, mean values for R_{max}, R_{min} and $R_{7.5}$ were re-determined in the same way at roughly two-monthly intervals.

Intracellular acid and alkali loading

Intracellular pH regulation was studied by inducing an acute intracellular acid load (for example, using the ammonium prepulse technique) or an acute intracellular base load (for example, using the Na-acetate pre-pulse technique) and then examining the subsequent recovery of pH_i back to control levels (cf. Roos & Boron, 1981). Ammonium pre-pulses were achieved with brief (3–5 min) extracellular exposures to 10 mM NH_4Cl. Acetate pre-pulses utilised somewhat longer (about 10 min) extracellular exposures to 40 mM Na acetate.

Calculation of acid-equivalent fluxes

In most of the present work, effects of drugs on acid-equivalent fluxes were estimated simply by comparing, in the same experiment, the rate of pH_i-recovery from an intracellular acid or alkali load, in the presence and absence of the drug. Comparisons were always made at a common pH_i, to eliminate possible variation in recovery rate due to pH_i dependent changes in intracellular H^+-buffering or acid-transport activation. In some experiments, however, the cell's background acid-loading rate was estimated (see eg. Figure 4). This was calculated from the equation: background loading $= \beta_i dpH_i/dt$, where β_i is intracellular buffering power, and dpH_i/dt is the rate of change of pH_i caused by an acid influx across the sarcolemma, or by *de novo* generation of acid (possibly metabolically) within the cell. The experiments examining background acid loading were performed on cells superfused with HEPES-buffered media (nominally free of CO_2/HCO_3^-). Under these conditions, intracellular buffering power was estimated from the equation: $\beta_i = -28\ pH_i + 222.6$. This is the best-fit linear equation describing the pH_i-dependence of intracellular, intrinsic buffering power in the guinea-pig ventricular myocyte (Lagadic-Gossmann *et al.*, 1992). The rate of change of pH_i (dpH_i/dt) was measured by computer as the least-squares regression line fitted to calibrated pH_i data sampled, at 0.5 s intervals, over a 1.5 min period (0.5 min. for experiments with

lactate addition/removal, see eg. Figure 6). This rate was then correlated with a single pH_i value, which was taken as the mid-point of the pH_i-range encompassed by the 1.5 min time period.

Statistics

Mean values are quoted ± standard error of mean (s.e. mean), with sample size (n). A t test was used to determine the effect of drugs on rate of pH_i-recovery, or on acid-equivalent flux. A P value of 0.05 or less was accepted to indicate statistical significance.

Results

Inhibition of cardiac NHE

In order to examine the effects of drugs on NHE, the experiments were performed in HEPES-buffered solutions, nominally free of CO_2/HCO_3^-. Under these conditions, pH_i recovery from an intracellular acidosis is mediated exclusively by acid extrusion on NHE (Lagadic-Gossmann *et al.*, 1992). The acid load can be imposed by the NH_4Cl pre-pulse technique (10 mM, see Methods). Possible contributions to acid extrusion from the Na^+-HCO_3^--symporter, activated by residual levels of CO_2/HCO_3^- (cf. Wu *et al.*, 1994) were excluded by the observation that the stilbene drug, DIDS (0.5 mM), an inhibitor of Na^+-HCO_3^- transport (Lagadic-Gossmann *et al.*, 1992; Dart & Vaughan-Jones, 1992), had no effect on pH_i recovery (pH_i recovery-rate, measured at 7.00, was inhibited in DIDS by 0 ± 4%, $n=4$; $P>0.05$). In contrast, amiloride, DMA and Hoe 694 (whose chemical structures are shown in Figure 1a(i) and 1a(ii)) all exerted a reversible, inhibitory effect on pH_i recovery, as illustrated in Figure 1b. All three drugs produced near 100% inhibition of pH_i recovery, measured at a test pH_i value of 6.90: amiloride (1.5 mM) 96 ± 2% inhibition, $n=11$; DMA (200 μM) 99 ± 1% inhibition, $n=6$; Hoe 694 (30 μM) 100 ± 0% inhibition, $n=6$. Thus Hoe 694, as shown previously, is a potent cardiac NHE-inhibitor.

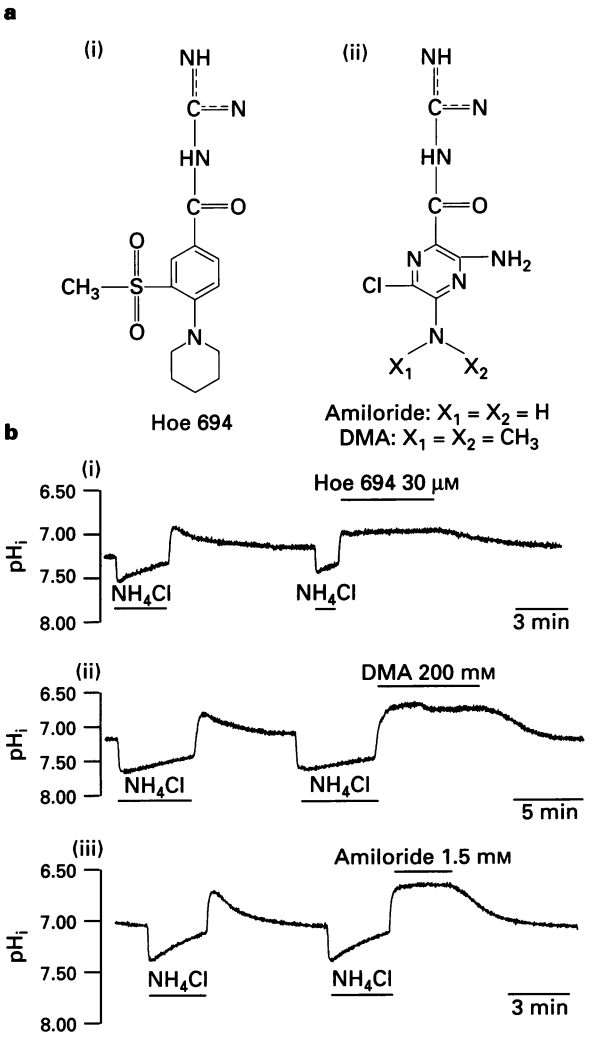

Figure 1 NHE inhibitors on pH_i recovery from acidosis: (a) structure of Hoe 694 (i) and amiloride or dimethyl amiloride (ii). (b) Experiments on three different cells showing, initially, pH_i-recovery from an intracellular acidosis induced by 10 mM NH_4Cl pre-pulse. Superfusates buffered with 20 mM HEPES (pH_0 7.40). Following the pH_i recovery, there was a second pre-pulse, producing acidosis in the presence of Hoe 694 (i), DMA (ii) or amiloride (iii).

Effect of Hoe 694 on other sarcolemmal acid-equivalent transporters

Current interpretation of the physiological effects of Hoe 694 in heart has relied upon the assumption that the drug selectivity blocks NHE thus disrupting the normal control of pH_i (Scholz *et al.*, 1993). We therefore examined the effect of the drug on the other sarcolemmal acid-equivalent carriers that contribute to pH_i regulation in the cardiac cell.

Na^+-HCO_3^- symport The first part of the trace shown in Figure 2a illustrates pH_i recovery from an acid load induced in nominally CO_2/HCO_3^--free conditions. This recovery is mediated by NHE. The second part of the trace shows pH_i recovery from acidosis in the presence of 5% CO_2/HCO_3^- (pH_0 7.4), a recovery mediated by both NHE and the Na^+-HCO_3^- symporter (Lagadic-Gossmann *et al.*, 1992). The histogram in Figure 2b shows net acid extrusion estimated at pH_i 6.93 ± 0.02 averaged for several experiments similar to that shown in Figure 2a. Extrusion in CO_2/HCO_3^--free conditions (HEPES) has been normalised to that measured, at the same pH_i and in the same experiment, in the presence of CO_2/HCO_3^-; the difference between these two values therefore equals the fraction of extrusion mediated through the symporter, and this is displayed in the fourth column of Figure 2b (HCO_3^--HEPES). The final part of the trace is shown in Figure 2a demonstrates that 30 μM Hoe 694 slows, but does not inhibit entirely, pH_i recovery under CO_2/HCO_3^--buffered conditions. The fraction of acid extrusion remaining in the presence of Hoe 694 is shown in the third column of Figure 2b. This is not significantly different ($P>0.05$) from the fraction mediated through the symporter (HCO_3^--HEPES, Figure 2b; about 35% of total net acid extrusion), implying that Hoe 694 inhibits NHE but not the symporter. In a separate set of experiments (not shown), we also found that pH_i recovery from acidosis in the presence of 5%CO_2/HCO_3^- plus 100 μM DMA (i.e. pH_i recovery mediated through the symporter; Lagadic-Gossmann *et al.*, 1992) was unaffected by the addition of 30 μM Hoe 694 ($n=4$) although in this latter case, possible competition for receptor-binding between Hoe 694 and DMA (Counillon *et al.*, 1993) could mean that receptor occupancy by Hoe 694 is reduced by up to 75% (assuming potencies for hypothetical binding to the symport similar to those shown in Figure 7 for inhibition of NHE). Nevertheless, the lack of *any* effect of Hoe 694 on pH_i recovery under these conditions, when combined with the previous data shown in Figure 2 is powerful evidence that Na^+-HCO_3^- symport is unaffected by the drug.

Cl^--HCO_3^- exchange This carrier is a sarcolemmal acid-loader, activated in the presence of HCO_3^- by a rise of intracellular pH, producing pH_i recovery from intracellular alkalosis. Such a recovery is shown in Figure 3a where the alkali load was induced by the acetate (40 mM) pre-pulse technique (see Methods). Hoe 694 (30 μM) exerted no inhibitory effect on

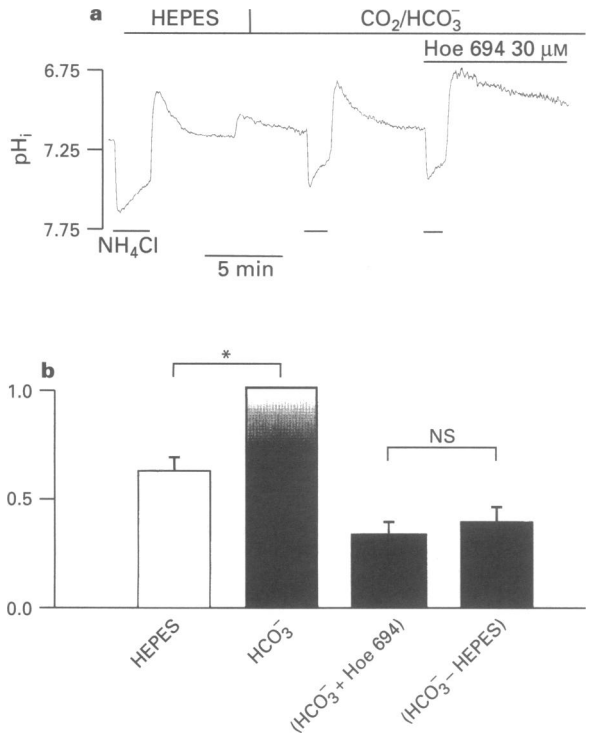

Figure 2 Effect of Hoe 694 on Na^+-HCO_3^- symport: (a) Top bar shows buffer system used in the superfusate (either 20 mM HEPES or 5%CO_2/HCO_3^-; pH_0 7.40). The periods of application of Hoe 694 (30 μM) and NH_4Cl (10 mM) are shown by the lower bars, immediately above and below the pH_i trace. (b) Histogram, showing net acid extrusion after acid loading estimated at pH_i 6.93±0.02 averaged for 7 experiments similar to that shown in (a). Note that the values for net acid extrusion have been normalized to that measured in 5%CO_2/HCO_3^- solution. Columns represent means±s.e. mean; *$P<0.001$; NS, not significant.

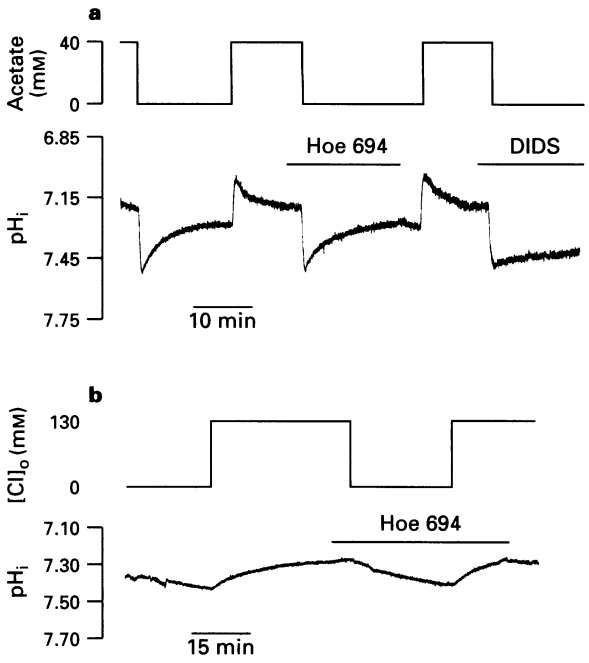

Figure 3 Effect of Hoe 694 and DIDS on the Cl^--HCO_3^- exchanger (AE). (a) Periods of superfusion of sodium acetate are shown at the top of the figure. Acetate-removal induces an intracellular alkali-load. Periods of superfusion of Hoe 694 (30 μM) and DIDS (0.5 mM) are shown by the bars above the pH_i trace. Superfusate buffered with 5%CO_2/23 mM HCO_3^- (pH_0 7.40). (b) Cl^-_0 removal (glucuronate-substituted) and re-addition reversibly stimulates the AE-carrier, producing a rise and fall of pH_i respectively. Periods of Cl^-_0-removal are shown at top of figure. Effect of 30 μM Hoe 694 on these pH_i-changes is assessed in the second half of the experiment.

pH_i-recovery from alkalosis, as shown in the middle section of Figure 3a. The pH_i recovery rate measured at pH_i 7.44±0.22, was changed in the presence of 30 μM Hoe 694 by −11±14%, $n=6$, an effect that was not statistically significant ($P>0.05$). In contrast, the recovery from alkalosis is slowed considerably by DIDS (shown at the end of the trace in Figure 3a; similar result seen in a second cell), consistent with the known stilbene-sensitivity of cardiac Cl^--HCO_3^- exchange (Vaughan-Jones, 1979; 1986; Alper, 1991).

Figure 3b illustrates another method of examining the Hoe 694-sensitivity of Cl^--HCO_3^- exchange. Cl^-_0-removal (replaced by glucuronate and gluconate) produces a slow alkalosis caused by Cl^- exit from the cell in exchange for HCO_3^- ion entry, while re-admitting Cl^-_0 causes a slow re-acidification of pH_i caused by Cl^- re-entry in exchange for HCO_3^- exit (Vaughan-Jones, 1979; Lagadic-Gossmann *et al.*, 1992; Xu & Spitzer, 1994). Hoe 694 (30 μM) again exerted no inhibitory effect on these pH_i changes ($n=5$); the rate of re-acidification upon Cl^-_0 re-addition was not significantly changed in the presence of the drug (11±14% rate-change, measured at pH_i 7.39±0.04, $n=5$; $P>0.05$).

The above results indicate that, at doses which inhibit Na^+/H^+ exchange maximally, Hoe 694 has no inhibitory potency for the Cl^--dependent acid-loaders in the cardiac cell.

Acid loading in HCO_3^--free conditions Even in the nominal absence of HCO_3^-, (HEPES buffer), there is evidence of basal acid-loading of the cardiomyocyte which will influence the control of pH_i. The cause of this loading is not known (it may, for example, be due to cellular CO_2 or lactic acid production, or it may be due to an unidentified sarcolemmal acid loading carrier), but when assessing effects of Hoe 694 on pH_i, it is important to establish if basal acid-loading is affected by the drug. As shown in Figure 4b, basal loading can be revealed by inhibiting NHE with 30 μM Hoe 694 and observing the subsequent acidification of pH_i. The acidification with Hoe 694 was similar to that observed when NHE was inhibited by removing extracellular Na^+ (Figure 4a; Na^+ replaced by N-methyl D-glucamine). The rate of acid-loading seen with Hoe 694 or in Na-free conditions was calculated ($-\beta_i \times dpH_i/dt$; see Methods) for several experiments and the results pooled in Figure 4c, for three pH_i ranges. It is apparent that basal loading in HEPES-media is small and that it declines somewhat as pH_i falls from 7.23 to 6.94. Acid-loading is not significantly different in Na-free and in Hoe 694. Since both manoeuvres expose acid loading by inhibiting NHE, this result suggests that Hoe 694 is not itself altering the basal acid-loading mechanism of the cell. The similar rate of acid-loading in Hoe 694 and in Na^+-free solution also suggests that reversal of Na^+-H^+ exchange in the latter condition is not large enough to enhance basal loading.

A lack of effect of Hoe 694 on basal acid-loading was confirmed by adding the drug in Na-free solution. One such experiment is shown in Figure 5, again using HEPES-buffered solutions (similar result in three other cells). In this case an ammonium pre-pulse had been performed in Na-free solution to impose an intracellular acid load. As expected, because of inhibition of NHE, there was little subsequent pH_i recovery until Na^+_0 was re-admitted. Figure 5 shows that addition of Hoe 694 exerted no effect on pH_i. With NHE inhibited, pH_i should be affected only by positive or negative changes in basal acid-loading. The lack of effect of Hoe 694 therefore indicates that basal acid loading is not influenced by the drug.

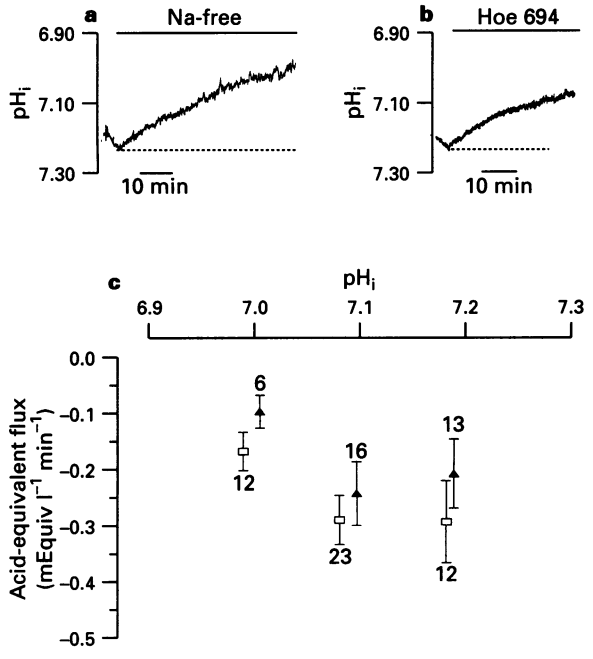

Figure 4 Comparison of background acid-loading, estimated using Hoe 694 (▲) or Na^+-free (□) conditions). All solutions were HEPES-buffered (20 mM, pH_0 7.40). (a) Acid loading, viewed following removal of extracellular Na^+ (NMDG-substituted); (b) acid-loading, viewed following NHE-inhibition with Hoe 694; (c) summary of data from several experiments similar to those shown in (a) and (b). Acid-loading ($\beta \times -dpH_i/dt$) is plotted as a function of pH_i (where β = intrinsic buffering power, see Methods for determination of β). Data have been binned according to pH_i 6.94–7.03; 7.04–7.13; 7.14–7.23. Sample size shown near each point. The differences in acid-loading in Na^+-free solution or in Hoe 694 are not significant ($P > 0.05$).

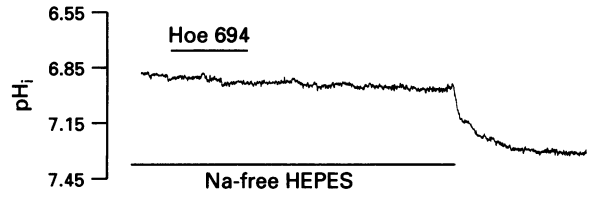

Figure 5 Effect of Hoe 694 on pH_i in Na^+-free (NMDG-substituted) solution. All solutions were 20 mM HEPES-buffered, pH_0 7.4. Hoe 694, 30 μM. An ammonium pre-pulse (10 mM) was performed, in the absence of extracellular Na^+ (NMDG-substituted), about 5 min before the beginning of the trace. This accounts for the low pH_i, seen in the first half of the experiment.

Lactate (monocarboxylic acid)-carrier Hoe 694 might conceivably affect pH_i by interfering with the uptake or release of lactic acid or other monocarboxylic acids from the cell. Figure 6 shows an experiment designed to demonstrate the effects on pH_i of activation of the sarcolemmal monocarboxylic acid carrier. A cardiac cell was superfused with an isotonic solution of 100 mM sodium citrate (pH_0 7.40) at 25°C. In the steady state under these conditions (see Wang *et al.*, 1994), pH_i becomes somewhat alkaline, HCO_3^-- and Cl^--dependent acid equivalent carriers are inactivated, and because of the high pH_i and relatively low temperature, Na^+-H^+ exchange is virtually inactive (see below). Addition of extracellular Na L-lactate (5 mM) produces a monotonic and rapid fall of pH_i, which is reversed upon lactate removal. These changes of pH_i are caused by sarcolemmal movement of lactic acid, much of which occurs via the monocarboxylic acid carrier (de Hemptinne *et al.*, 1983; Wang *et al.*, 1993). This is confirmed as

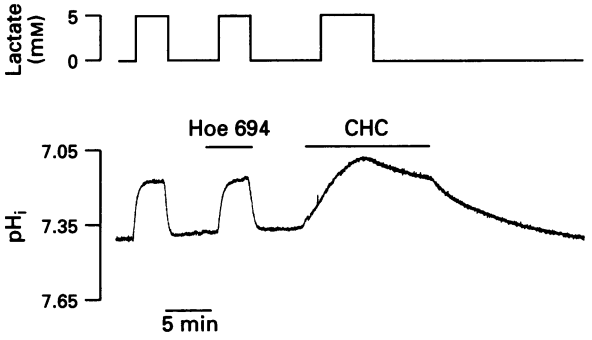

Figure 6 Effect of Hoe 694 and cyano-hydroxycinnamate (CHC) on the lactate-proton symporter. See text for details of the superfusate composition (essentially, an isotonic sodium citrate solution, nominally free of CO_2/HCO_3^-; pH_0 7.40; temperature 25°C). Periods of application of extracellular L-sodium lactate are shown at the top of the trace. Hoe 694, 30 μM; CHC, 3 mM.

shown previously (Wang *et al.*, 1994), by the observation that CHC (α-cyano-4-hydroxycinnamate), an inhibitor of the carrier (Poole & Halestrap, 1993), attenuates considerably the rapid fall of pH_i upon lactate-addition, and greatly slows its recovery upon lactate-removal (similar result in six other cells). Figure 6 shows that 30 μM Hoe 694 exerted virtually no effect on the lactate-induced fall of pH_i which, when averaged over the first 30 s, was reduced by 6 ± 17%, $n = 7$. This shows that Hoe 694 has no effect on the monocarboxylic acid carrier. The virtual lack of effect of Hoe 694 on pH_i recovery following lactate-removal (identical result in 6 other cells), also confirms that under these particular conditions, the majority of pH_i recovery is via the lactate carrier and not via NHE.

Pharmacological profiling of cardiac NHE with NHE-inhibitors

Potency series for NHE inhibitors The previous section indicates that Hoe 694 is a highly selective NHE inhibitor, not affecting the other carriers involved in pH_i regulation in the cardiomyocyte. The experiments shown in Figure 7 compare the potency of the Hoe 694 for cardiac NHE with that of amiloride and its derivative, DMA. With each drug, there was dose-dependent inhibition of pH_i recovery from an acid load (loads induced by ammonium pre-pulse while using HEPES-buffered solutions; recovery is thus due to activation of NHE). Specimen results are illustrated for DMA and Hoe 694 in Figure 7a and b. Results with each of the three NHE inhibitors are summarised in the graph shown in Figure 7c. Comparison of dose-response curves for the three inhibitors gives a potency series for cardiac NHE inhibition of: Hoe 694 ≃ DMA > > amiloride.

Hoe 694 has similar potency to that of DMA, one of the high-affinity amiloride analogues. Table 1**A**, lists this potency series and compares it with the pharmacological profiling (Table 1**B**), using the same three drugs, of the cloned NHE 1–3 isoforms when expressed in mutant fibroblast cells devoid of native NHE (Counillon *et al.*, 1993). It is apparent that the potency series obtained in the present work for native cardiac NHE matches that for cloned and expressed NHE-1. The series for NHE 2 and 3 are very different, in that Hoe 694 is the least potent inhibitor, the opposite of that found for cloned NHE-1 and for native cardiac NHE.

Potency values for NHE inhibitors The potency values determined from Figure 7c (concentration of drug required for 50% inhibition of NHE, defined here as apparent K_i are listed in Table 1C. For comparison, the potencies (K_i) determined previously (Counillon *et al.*, 1993) for the drugs acting on cloned NHE1–3 expressed in mutant fibroblasts are also listed (Table 1**E**). The apparent K_i values determined for native,

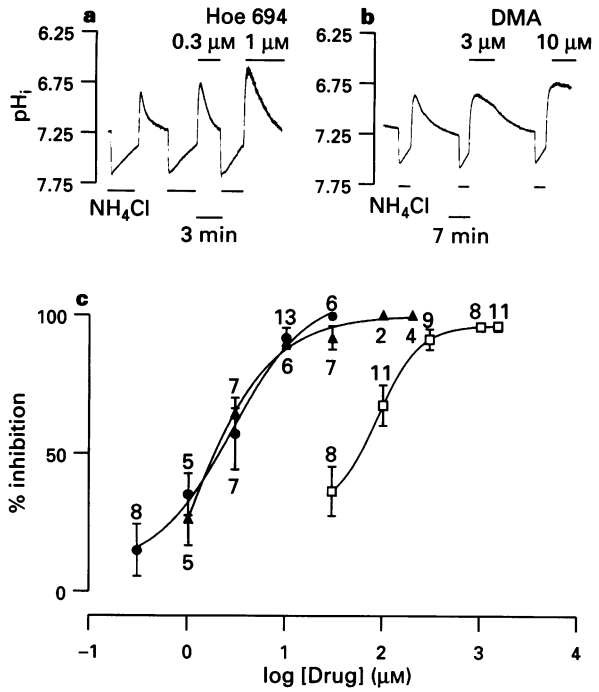

Figure 7 Dose-dependent inhibition of NHE by Hoe 694 (●), DMA (▲) and amiloride (□). Experimental protocol showing effects of different doses of Hoe 694 and DMA in two separate cells. Bars beneath pH_i traces show period of exposure to 10 mM NH_4Cl. All superfusates buffered with 20 mM HEPES, pH_0 7.40. (c) Normalised dose-response curves, showing percentage inhibition of pH_i recovery (measured at pH_i 6.95) plotted as a function of $\log_{10}$ [drug-concentration]. Curves drawn through points are best-fit Michaelis-Menten curves (correlation coefficients > 0.99), with IC_{50} values as listed in Table 1C. Curves fitted using Graph-plot (GraphPAD InPlot software).

cardiac NHE are considerably higher than those listed for cloned NHE-1, but this is to be expected because of competition of Hoe 694, amiloride or DMA with Na^+_0, for binding to the cardiac NHE protein (see Counillon *et al.*, 1993; all three drugs bind competitively). In the present work, such competition will raise the apparent K_i for each drug (Na^+_0 = 150 mM). The original pharmacological profiling of cloned NHE isoforms (Counillon *et al.*, 1993; Table 1E) was conducted in the virtual absence of Na^+_0 (100 μM) so that Na^+_0-effects on drug-binding were negligible. Apparent values for drug potency ($K_{i\text{-apparent}}$) in the presence of Na^+_0-competition are related to the real potency values ($K_{i\text{-absolute}}$; with no Na^+-competition) in accordance with the Michaelis Menten binding equation:

$$K_{i-\text{apparent}} = K_{i-\text{absolute}} \times (1 + [Na]_o / K^{Na}{}_{0.5}) \qquad (1)$$

where $K^{Na}{}_{0.5}$ = the NHE affinity constant for Na^+_0 (cardiac NHE is half saturated at 10 mM Na^+_0; e.g. Frelin *et al.*, 1984; Ellis & MacLeod, 1985), and Na^+_0 = 150 mM. Using this equation, $K_{i\text{-absolute}}$ values were calculated from our present data and are listed in Table 1**D**. It is clear that these potency values now match very closely those determined previously for the cloned isoform, NHE-1. In contrast, potency values for Hoe 694 acting on cloned NHE 2 and 3 are 40–500 times higher, respectively, than that found here for native, cardiac NHE.

Discussion

Selectivity of Hoe 694 for NHE

The benzoyl guanidine series of drugs from Hoechst is a new class of NHE inhibitor, not strictly related structurally to amiloride (for chemical structures, see Figure 1a(i) and (ii)). The potential of the Hoe drugs as cardioprotective agents during myocardial ischaemia and reperfusion is believed to be derived from their NHE-inhibiting properties (e.g. Scholz *et al.*, 1993). But, as with all new classes of drug, the potential of the Hoe benzoyl guanidines for targeting sites other than NHE must be assessed before they can be described as truly NHE-selective. The present work is the first to establish that Hoe 694 at doses that are maximal for inhibiting cardiac NHE has no pharmacological activity on any other known sarcolemmal transporter involved in the transport of acid-equivalents, and hence has no effect on mechanisms other than NHE which regulate intracellular pH in the cardiomyocyte. These other mechanisms include Na^+-HCO_3^- symport, Cl^--dependent acid-loading mechanisms (such as Cl^--HCO_3^- exchange), and the monocarboxylic acid carrier. Finally, Hoe 694 does not alter the properties of basal acid-loading in the cardiac cell, although the precise mechanism of this basal-loading remains to be determined.

Table 1 Potency series and potency values for the three NHE inhibitors

Native cardiac NHE (present work)

A

DMA ≃ Hoe 694 > > amiloride

C

Apparent K_i (μM)		
Hoe 694	DMA	amiloride
2.05	1.96	87.3

D

Absolute K_i (μM)		
Hoe 694	DMA	amiloride
0.13	0.12	5.5

→

Cloned and expressed NHE (Counillon *et al.*, 1993)

B

NHE1 DMA ≃ Hoe 694 > > amiloride
NHE2 DMA > amiloride ≃ Hoe 694
NHE3 DMA > amiloride > Hoe 694

E

	Absolute K_i (μM)		
	Hoe 694	DMA	amiloride
NHE1	0.16	0.1	3
NHE2	5	0.7	3
NHE3	650	11	100

A, potency-series of inhibitors used in this study (data from Figure 7); **B**, potency series of same three inhibitors for inhibition of Na^+ uptake through cloned NHE-isoforms expressed in mutant fibroblasts (data from Counillon *et al.*, 1993). **C**, potencies (apparent K_i) for the three inhibitors used in this study (data from Figure 7); **D**, estimates of absolute potency values (absolute K_i) for the inhibitors, derived from data shown in panel C using equation 1 in the text (which makes allowance for binding-competition of inhibitors with Na_o^+); **E**, absolute potency values (absolute K_i) determined previously for cloned NHE-isoforms expressed in mutant fibroblasts (data taken from Counillon *et al.*, 1993). Note that the absolute potencies estimated for the inhibitors used in the present work on native cardiac NHE, match closely those determined previously for the cloned NHE-1 isoform.

Whether Hoe 694 affects other non pH-related mechanisms is not known. For example, we do not know if, like amiloride, it also inhibits Na-Ca exchange (Kimura *et al.*, 1986), although Hendrikx *et al.*, (1994) suggest it may not. Effects on other fundamental biochemical systems also remain to be tested. Our data show, however, that as far as pH_i control in the heart is concerned, Hoe 694 does seem to be highly NHE-selective which, in turn, suggests that its cardioprotective properties may be secondary to the inhibition of cardiac NHE.

Functional isoform in heart is NHE 1

Our results indicate that the method, proposed by Counillon *et al.*, (1993), of identifying native NHE in a cell type by observing the differential inhibitory effects of various NHE inhibitors including Hoe 694, can be applied successfully to the cardiac cell, where the results point clearly to the dominant, functional expression of NHE 1. One obvious problem with the NHE inhibitor-profiling technique is that it may be difficult to resolve accurately the co-expression of more than one NHE isoform. If, for example, two isoforms are present each with its function being expressed equally in terms of effects on pH_i regulation, then the NHE profiling technique will produce an equivocal answer, with a potency series and K_i values roughly midway between the two individual NHE profiles. Inspection of the mixed profile may, nevertheless, give a clue as to the component isoforms, and if these components are known already from the molecular biology of the tissue, then it may be possible to use the pharmacological profile to predict the fractional contribution of each isoform to the control of pH_i. In the present case, however, the data are in line with the NHE 1 profile which suggests an essentially homogeneous isoform population in ventricular cardiac cells. This conclusion is in good agreement with the failure, so far, of molecular biological techniques to detect significant quantities of mRNA for cardiac isoforms other than NHE 1 (Fliegel *et al.*, 1993). While we do not exclude entirely the expression of other isoforms, their functional influence on pH_i control must be small, indicating that they are either physiologically inactive or expressed at very low density.

Finally, since a pharmacological profile of cloned NHE 4 using NHE inhibitors, has yet to be produced one might ask if the present data can exclude a significant presence of this isoform in cardiac cells. The answer would appear to be that it can be excluded, on the grounds that data available so far for NHE 4 indicate it is essentially insensitive to amiloride (Yun *et al.*, 1995). The amiloride-sensitivity detected for cardiac NHE in the present work is at the top of the list of amiloride sensitivities for the NHE isoforms (cf. Table 1E). Thus it is extremely unlikely that cardiac NHE contains a significant fraction of NHE 4.

In conclusion, the use of Hoe 694 alongside other more classical NHE-inhibitors (amiloride analogues) provides pharmacological evidence for the functioning of NHE-1 during pH_i-control in mammalian ventricular myocytes. In addition, Hoe 694 is highly selective for NHE over other acid-transporting carriers, thus providing further evidence that NHE is likely to be the pharmacological target mediating the cardioprotective properties of the drug.

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