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Intracellular pH alterations induced by tacrine in a rat liver biliary epithelial cell line

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1 The effects of tacrine (THA) on intracellular pH (pH_i) were examined in a rat liver biliary epithelial cell line (RLEC) in HEPES-buffered medium. pH_i was recorded using the pH-sensitive fluoroprobe, carboxy-SNARF-1 (carboxy-seminaphtorhodafluor).

2 In the steady state, short-term exposures to THA resulted in alkalinization and re-acidification at 0.1 and 0.25 mM. Following a 24 h-treatment, no significant difference in pH_i could be detected at 0.1 and 0.25 mM THA, whereas at 0.05 mM, pH_i was slightly more acid (7.17 ± 0.02, $n=16$ *versus* 7.21 ± 0.02, $n=24$ [control]).

3 In control and short-term treated cells, intracellular intrinsic buffering power (β_i) increased roughly linearly as pH_i decreased. This dependence was not seen following long-term treatment. In all cases, β_i was increased by THA (by 1.6 to 3.5 fold).

4 Following an acid load (induced by 20 mM NH_4Cl removal), pH_i recovery in RLEC relied upon Na^+/H^+ exchange. A short-term treatment (0.25 mM THA) did not affect total acid extrusion. In contrast, a 24 h-treatment with 0.05 mM THA reduced it (by $\approx 36\%$ at a pH_i of 6.73) while at 0.25 mM, a large increase was detected (by $\approx 109\%$ at a pH_i of 6.75).

5 In Na^+-free medium, THA (0.25 mM) still induced an alkalinization in the steady state. Following an acid load, THA stimulated a Na^+-independent acid efflux in a dose-dependent manner, inhibitable by α-cyano-4-hydroxy cinnamate (CHC, 4 mM) but not by quercetin (0.125 mM).

6 In conclusion, this work demonstrates that THA affects pH_i in RLEC, through a decrease in Na^+/H^+ exchange and an increase in β_i. Stimulation of a CHC-inhibitable, Na^+-independent acid efflux is also detected.

Keywords: Intracellular pH regulation; acid extrusion; tacrine; liver non-parenchymal epithelial cell; intrinsic buffering power; sodium-hydrogen exchange; quercetin; α-cyano-4-hydroxy cinnamate

Abbreviations: RLEC, rat liver biliary epithelial cells

Introduction

Alzheimer's disease is a progressive, degenerative dementia characterized by decreased cognitive functions with associated decline in cholinergic transmission. Tacrine (1,2,3,4-tetrahydro-9-aminoacridine; THA) is a centrally active acetylcholinesterase inhibitor that has been developed for the treatment of this disease (Farlow *et al*., 1992; Knapp *et al*., 1994). Unfortunately, the clinical use of this molecule has been demonstrated to induce hepatotoxicity in about 30–50% of the treated patients, as evidenced by an increase in the serum alanine aminotransferase (ALT) activity (Forsyth *et al*., 1989; O'Brien *et al*., 1991; Watkins *et al*., 1994). However, the mechanisms underlying the hepatotoxicity of tacrine remain to be fully established.

Until recently, it was supposed that the oxidative metabolism of tacrine, mainly *via* cytochrome P450 1A2, was responsible for the deleterious effects of this molecule, due to the production of reactive metabolites (Madden *et al*., 1993). However, general observations dealing with this hypothesis do not support such a conclusion. Among all the data against, it has been found that tacrine is equally cytotoxic to rat hepatocytes and HepG2 human hepatoma cells, known to lack CYP1A2 activity (Viau *et al*., 1993). Similarly, we have shown that the viability of a rat liver non-parenchymal epithelial cell line (RLEC) was also affected upon tacrine exposure despite the absence of any metabolism of this molecule in this cell line (Lagadic-Gossmann *et al*., 1998). This pointed to an effect of the molecule *per se*. Recent work by Berson *et al*. (1996) and Robertson *et al*. (1998) have shown that a mitochondrial dysfunction is elicited by tacrine. However, as raised by the latter group, mitochondrial dysfunction might not be the only factor involved, since the clinical manifestations of tacrine do not resemble those typically associated with mitochondrial cytopathies (Feuer & de la Iglesia, 1996). Moreover, in our hands, although we have clearly shown an effect of THA on the intermediary metabolism of hepatocytes, this could rather represent a late event in the cascade leading to cell death. Indeed, alterations in the cell membrane (as revealed by lactate dehydrogenase leakage) were detected prior to decreases in ATP (Lagadic-Gossmann *et al*., 1998). Therefore, other factors should be considered.

Maintenance of cytoplasmic pH (pH_i) within a narrow physiological range is vital to normal cell function (Strazzabosco & Boyer, 1996). Nevertheless, this parameter can vary in response to diverse events (e.g. following stimulation of intermediary metabolism or upon hormone application). Under homeostatic conditions, these variations are quickly

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normalized by several membrane mechanisms. However, in some pathological states (e.g. ischaemia-reperfusion sequence) or following exposure to xenobiotics (e.g. cadmium or ethanol), the activity of these mechanisms is affected leading to long-lasting pH_i changes (Koizumi *et al*., 1994; Benedetti *et al*., 1995a; Strazzabosco & Boyer, 1996) and hence to alterations to cell function and ultimately cell death. With respect to tacrine, this weak base has been suggested to affect the mitochondrial pH gradient through a protonophoric action, thus resulting in mitochondrial dysfunction (Berson *et al*., 1996). In this context, we hypothesized that tacrine might also induce cytoplasmic pH (pH_i) variations.

In this study, the effects of tacrine on pH_i homeostasis were investigated in cultured RLEC using the intracellular fluorophore carboxy-SNARF-1. Our data were obtained in HEPES-buffered medium with the aim of evaluating the effects of tacrine on the activity of the ubiquitous Na^+/H^+ exchange, known to regulate pH_i following an intracellular acid challenge. Special attention was paid to this exchange since it has been described to play an important role in the cytotoxic processes when pH_i alterations are involved (Karmazyn & Moffat, 1993; Benedetti *et al*., 1995b; Boyle *et al*., 1997). We decided to perform experiments in RLEC because of three main reasons: (i) this cell line originated from primitive bile ductules in the liver, the organ described as the most susceptible to tacrine toxicity, and we have recently shown that RLEC were as sensitive as hepatocytes to the adverse effects of tacrine (Lagadic-Gossmann *et al*., 1998); (ii) these cells do not metabolize THA so that only the effects of tacrine *per se* were assessed (Lagadic-Gossmann *et al*., 1998); and (iii) isolated biliary epithelial cells have been shown to possess several ion membrane transporters involved in pH regulation, cell volume control and bile formation (Elsing *et al*., 1996; Strazzabosco *et al*., 1996).

Methods

Cell isolation and culture

RLEC, thought to derive from primitive bile ductules, were obtained by trypsinization of the livers of 10-day-old Sprague-Dawley rats as described elsewhere (Morel-Chany *et al*., 1978) and monolayers of RLEC were used between passages 10 and 25. They were seeded on 25 mm-diameter glass coverslips and cultured in Williams' E medium (Eurobio, France) supplemented with 10% foetal calf serum (Dominique Dutscher SA, Brumath, France) and (per ml) 5 IU penicillin and 5 μg streptomycin. RLEC were used 1 week after reaching confluency.

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.

Experimental solutions

HEPES-buffered solution contained (in mM): NaCl 134.8, KCl 4.7, $MgCl_2$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 1.0, glutamine 2, glucose 10; HEPES (N-2-hydroxyethylpiperazine-N′-2-ethanesulphonic acid) 10, pH adjusted to pH 7.4 at 37°C with NaOH. In Na^+-free medium, NaCl was replaced with 134.8 mM N-methyl-D-glucamine and the pH adjusted to 7.4 at 37°C with HCl. When ammonium chloride (NH_4Cl 20 mM; Sigma Chemical Co., St. Louis, MO, U.S.A.) was used, 20 mM NaCl was removed from the medium in order to avoid any change of osmotic force. NH_4Cl was added 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). Ethyl isopropyl amiloride (EIPA, 50 μM; Sigma; an inhibitor of Na^+/H^+ exchange; Vigne *et al*., 1983), quercetin (125 μM; Sigma; an inhibitor of $Lactate^-$-H^+ cotransport; Rosenberg *et al*., 1993) and α-cyano-4-hydroxy cinnamate (CHC 4 mM; Sigma; an inhibitor of $Lactate^-$-H^+ cotransport; Rosenberg *et al*., 1993) were all dissolved in dimethylsulphoxide (DMSO) before addition to the HEPES-buffered solution (DMSO concentration $<0.1\%$). Nigericin (Sigma) calibration solutions used in this study have been described elsewhere (Lagadic-Gossmann *et al*., 1992).

Drug application protocol

Tacrine hydrochloride hydrate was purchased from Sigma. It was first prepared as a stock solution (20 mM) either in culture medium (long-term treatments) or HEPES-buffered medium (short-term treatments) and kept at 4°C for up to 15 days. Media with THA at the test concentrations (0.25, 0.1 and 0.05 mM) were prepared just prior to the experiments. The concentration of 0.25 mM was chosen since we previously demonstrated it to be close to the IC_{50} of THA (equal to 0.278 mM) towards lactate dehydrogenase (LDH) release in RLEC after a 24-h treatment. The other two concentrations were determined as non toxic after 24 h (Lagadic-Gossmann *et al*., 1998).

THA was applied to RLEC for 15–30 min (short-term treatment) or 24 h (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. In this study, pH_i recoveries from THA-treated cells were compared to those estimated in independent control non-treated cells. Only in a few cases, especially when studying short-term effects, pH_i recoveries in the absence and presence of THA were obtained from the same cells (considering the fact that two consecutive recoveries were found to be identical in RLEC; not shown). In the course of our experiments, we decided to pool results obtained from all control cells, since no culture time-dependent change in pH_i recovery was observed in this group.

Measurement of pH_i

The pH_i of RLEC cultured on glass coverslips was monitored using the pH-sensitive fluorescent probe, carboxy-SNARF-1 (carboxy-seminaphtorhodafluor; 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 acetoxy-methyl ester for 20 min at room temperature just prior to performing the pH_i recording. Under our experimental conditions, we verified that SNARF was not cytotoxic (using the Trypan blue exclusion test).

SNARF-loaded cells were placed in a continuously perfused recording chamber (at a temperature of 36 ± 1°C) mounted on the stage of an epifluorescence microscope (Nikon Diaphot). RLEC were then excited with light at 514 nm and fluorescence from the trapped probe was measured at 590 and 640 nm. The necessary monochromator and photometers to produce and detect the fluorescence were part of a Photon Technology International (PTI; NJ, U.S.A.) DeltaRAM system, and the software systems to control the monochromator and both acquire and process the data were also supplied by PTI. Emitted fluorescence signals were recorded every 12 s and originated from a small area of the coverslip representing

approximately 10 cells in the field of view. When using cells treated for 24 h with 0.25 mM THA (a treatment known to induce some cytotoxic effects in RLEC; see above), one might suppose that some of the cells in the recording field may be damaged; however, based on the fact that fluorogenic esterase substrates (such as SNARF-AM) and their fluorescent products rapidly leak out (within seconds) from dead or damaged cells (Haugland, 1996), the recorded fluorescence was most likely related to intact cells (especially as recording was always started following a 8–10 min lag period after dye loading, thus allowing complete dye leakage from damaged cells). Background fluorescence intensity values (Fi) at the two emission wavelengths (590 and 640 nm) were obtained during excitation at 514 nm of unloaded cells after 3–5 min perfusion with HEPES-buffer. At this excitation wavelength, we noted that Fi_{590} and Fi_{640} were mainly due to the instrument since similar background Fi values were obtained using coverslips without any seeded cell. At the beginning of any experiment, values for Fi_{590} and Fi_{640} were 12.6 ± 0.7 (s.e.mean) and 4.4 ± 0.2 (s.e.mean)-fold higher than background Fi values detected at these two emission wavelengths, respectively. Background fluorescence was not affected by THA. Dye leakage over the subsequent experimental (20–40 min) period, measured as the percentage decline in Fi_{590} and Fi_{640}, was around 30–35%. In preliminary experiments, we verified that, under our experimental conditions, no phototoxicity occurred (using the Trypan blue exclusion test) and that photobleaching contributed little to the fall in Fi; indeed, Fi values fell at similar rates when Fi measurements were made every (a) 12 s and (b) after 0 and 20–40 min. The emission ratio 640/590 (corrected for background fluorescence) obtained from intracellular SNARF was calculated and converted to a linear pH scale using *in situ* calibration data obtained by the nigericin technique described elsewhere (Thomas *et al*., 1979; Buckler & Vaughan-Jones, 1990).

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, 1990; Lagadic-Gossmann *et al*., 1992). Briefly, a stepwise reduction of external NH_4Cl (from 20 mM) was applied to selected cells. Each reduction in NH_4^+ resulted in the generation of intracellular H^+, due to the dissociation of NH_4^+ into H^+ ions and NH_3, with subsequent efflux of NH_3. The resultant changes (Δ) in pH_i were used to estimate β_i as follows:

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

where $[NH_4^+]_i = ([H^+]_i \times [NH_4^+]_o)/[H^+]_o$. In this latter equation, $[NH_4^+]_i$ and $[NH_4^+]_o$ were intracellular and extracellular ammonium ion concentrations, respectively; $[H^+]_i$ and $[H^+]_o$ were intracellular and extracellular proton concentrations, respectively. The experiments were carried out in the absence of extracellular Na^+ in order to prevent (or at least to limit in the case of THA treatments) acid extrusion and in the presence of barium (1 mM) to reduce NH_4^+ efflux through potassium channels (Vaughan-Jones & Wu, 1990).

Calculation of sarcolemmal acid efflux

Details of the method for calculating acid efflux (J^e_H) during pH_i recovery in cells have been described previously (Lagadic-Gossmann *et al*., 1992). Briefly, acid efflux was estimated using the following equation: $J^e_H = \beta_T \cdot dpH_i/dt$, 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 the use of one of the equations (1) or (2) in the Results (these are empirical descriptions of the dependence of β_i upon pH_i under control conditions and upon short-term THA-treatments). When effects of long-term THA-treatments on J^e_H were studied, we used the constant values (depending on experimental conditions) given in the Results, since we found that β_i then became insensitive to pH_i under such conditions.

Statistics

All data are quoted as mean $\pm$ standard error of mean (s.e.mean) along with the number of observations, n, corresponding, if not otherwise stated, to the number of separate cultures used. Cells from different passages (at least 2) were used for all the protocols carried out. Student's t-test or analysis of variance followed by Newman-Keuls test were used to test the effects of THA. Linear regression analysis was performed by the method of least square. Differences were considered significant at the level of $P < 0.05$.

Results

Effects of tacrine on steady-state pH_i

The effects of different concentrations of tacrine on steady-state pH_i were evaluated in rat liver biliary epithelial cells following short-term (15 min) and long-term (24 h) exposures. Figure 1a shows a representative pH_i recording obtained using carboxy-SNARF-1 in RLEC superfused with a HEPES-buffered solution. Under these conditions, the steady-state pH_i of these cells was 7.21 ± 0.02 ($n = 24$). Upon short-term application of tacrine (0.25 mM), a biphasic change was observed with, at first, a rapid alkalinization followed by a slow intracellular acidification. As shown in Figure 1b,c, both changes (i.e. maximum of alkalinization and initial rate of acidification) increased with increasing THA concentration, the effects at 0.25 mM being significantly different from those observed at 0.05 mM. At this latter concentration, it should be noted that whereas the alkalinization was absent, a significant acidic shift was recorded ($P < 0.001$ when compared to control, steady-state conditions, $n = 6$). Following a 24 h-treatment, no significant difference in steady-state pH_i could be detected at the highest THA concentrations tested when compared to control conditions (7.21 ± 0.02 [$n = 18$] and 7.20 ± 0.01 [$n = 18$] at 0.1 and 0.25 mM THA, respectively, *versus* 7.21 ± 0.02 [$n = 24$]). In contrast, upon a 24-h treatment with 0.05 mM THA, RLEC exhibited a slightly, but significantly, more acid steady-state pH_i compared to all other conditions ($pH_i = 7.17 \pm 0.02$ [$n = 16$]; $P < 0.05$).

Intrinsic intracellular buffering power, β_i

In a next set of experiments, we tested the effects of tacrine on intracellular intrinsic H^+ buffering power (β_i) in RLEC. β_i was estimated over the range 6.5–7.6 using the stepwise removal of external NH_4Cl (from 20 mM). The resulting estimates of β_i at various values of pH_i are illustrated in Figure 2. It is clear from Figure 2a that: (i) β_i increased as pH_i decreased; (ii) an acute treatment with tacrine (applied at 0.25 mM a few minutes prior to the beginning of the procedure used to estimate β_i) enhanced β_i over the whole pH_i range tested. When fitted by a least

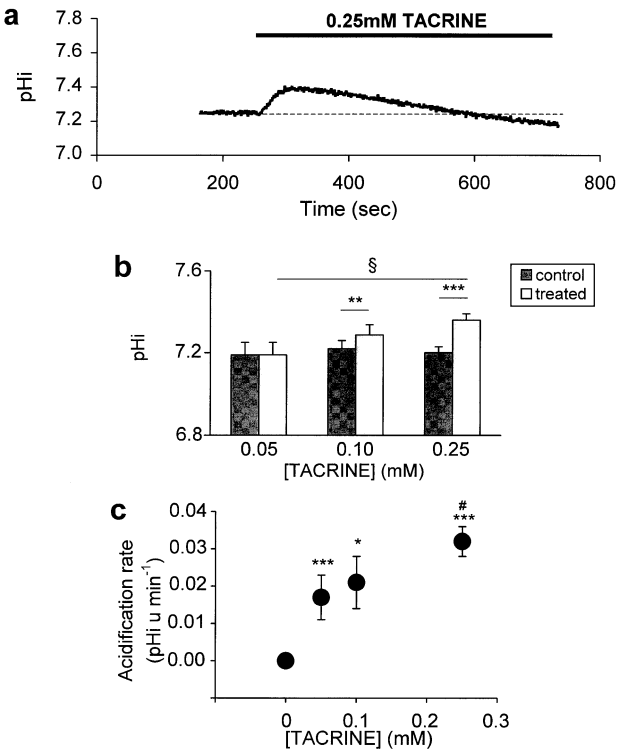

Figure 1 Effects of short-term applications of tacrine on intracellular pH (pH_i) recorded in RLEC superfused with HEPES-buffered solution. (a) Shows a typical experiment in which the effect of tacrine (0.25 mM) was tested on the steady-state pH_i. Fluorimetric pH_i recording was obtained by use of carboxy-SNARF-1. The graph in (b) shows the maximum alkaline pH_i elicited upon different concentrations of tacrine. ⟪Control⟫ corresponds to steady-state pH_i recorded prior to THA exposure while ⟪treated⟫ refers to maximum alkaline pH_i recorded in the presence of THA. Data are given as mean ± s.e.mean for $n = 6$, 4 and 11 similar experiments at the concentrations of 0.05, 0.10 and 0.25 mM tacrine, respectively. **($P < 0.01$) and ***($P < 0.001$) indicate significant difference between ⟪treated⟫ and ⟪control⟫ (paired t-test). §($P < 0.001$; t-test): significant difference between maximum pH_i recorded in the presence of 0.25 mM and that in 0.05 mM THA. In (c), the initial rate of re-acidification is plotted *versus* tacrine concentrations (u: unit). The control value plotted *versus* [THA] = 0 mM refers to the rate of acidification prior to addition of THA and is equal to zero. *($P < 0.05$) and ***($P < 0.001$): significant difference between ⟪control⟫ and ⟪treated⟫ (paired t-test); #($P < 0.002$): significant difference between 0.25 mM and 0.05 mM THA (t-test).

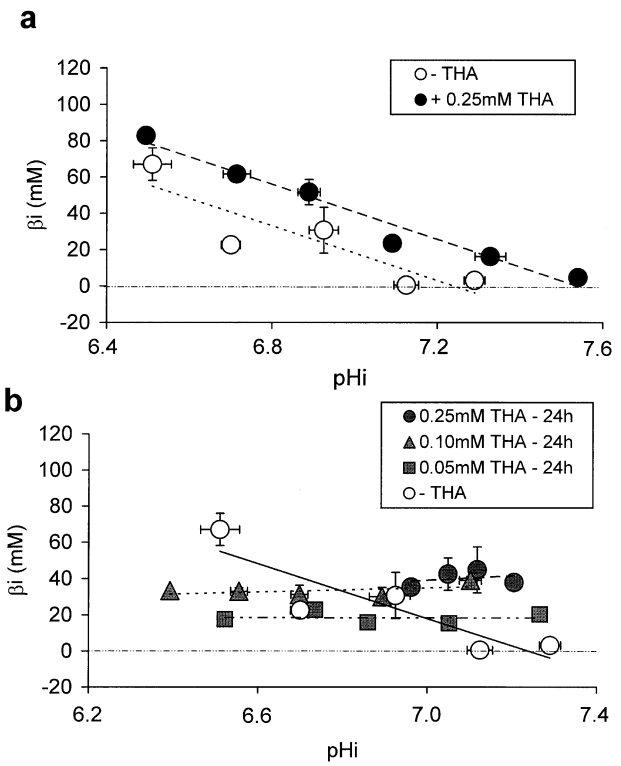

Figure 2 Average effects of tacrine (THA) on pH_i-dependence of intracellular intrinsic buffering power (β_i). (a) Shows pH_i-dependence of β_i in control, untreated cells (−THA; $n = 6$ separate experiments) and in cells following a short-term treatment with 0.25 mM THA (+0.25 mM THA; $n = 6$). In (b), the effects of different concentrations of THA were tested on this parameter following a 24 h-treatment ($n = 5$ at 0.05 mM [0.05 mM THA−24 h]; $n = 6$ at 0.10 [0.10 mM THA−24 h] and 0.25 mM [0.25 mM THA−24 h]; control pH_i-dependence of β_i (−THA) was replotted in (b) in order to ease comparison. 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: 6.2–6.4; 6.4–6.6; 6.6–6.8; 6.8–7.0; 7.0–7.2; 7.2–7.4; 7.4–7.6. In each group, data were fitted by a linear least-square regression analysis.

square linear regression, estimates of β_i could be described by the following equations:

$$\beta_i = 546.1 - (75.4 \times pH_i) \quad (1)$$

for the control group ($r^2 = 0.78$; $n = 6$; $P < 0.047$) and

$$\beta_i = 573.1 - (76.0 \times pH_i) \quad (2)$$

for the THA-exposed group ($r^2 = 0.97$, $n = 6$; $P < 0.001$). These equations indicate that, at e.g. pH_i 7.1, β_i will be ≈11 mM (control) *versus* 33.5 mM (+THA). Following a 24 h-treatment with THA, an increase of β_i was observed only in the steady-state pH_i range, i.e. 7.0–7.3, especially at the concentrations of THA of 0.10 and 0.25 mM (Figure 2b). From this figure, it also appeared that, following such long-term treatments, β_i became independent of pH_i. Therefore, the following mean β_i values, rather than equation (2), were used to calculate acid effluxes in the different long-term treated groups: 17.7 ± 1.1 mM (THA 0.05 mM; $n = 12$ determinations from m = 5 coverslips); 33 ± 1.8 mM (THA 0.10 mM; $n = 23$, m = 6); 39 ± 3.4 mM (THA 0.25 mM; $n = 18$, m = 6). Finally, considering these β_i values, it was worth noting that β_i was significantly lower at 0.05 mM THA compared to the other two concentrations ($P < 0.001$), while at the highest concentration used (0.25 mM), this parameter was not significantly increased compared to that determined at 0.10 mM THA.

Effects of tacrine on pH_i regulation following an intracellular acidification

Control conditions The activity of the membrane acid-extruders is usually assessed following their activation; this is realized by acid-loading the cells using the NH_4^+ pre-pulse method (Boron & De Weer, 1976). In Figure 3a, the RLEC were acid-loaded twice; the first part of the recording shows a representative control pH_i recovery obtained following an acid load induced by addition and then removal of 20 mM NH_4Cl. Prior to evaluating the effects of tacrine, we first demonstrated that in HEPES-buffered medium, only Na^+/H^+ exchange was functional in the cell line used. So, EIPA (50 μM; Figure 3a: second pulse), a derivative of amiloride known to inhibit this exchange, as well as extracellular Na^+ removal (Figure 3b) both resulted in an inhibition of pH_i recovery. In Figure 4a, the cells were, as in Figure 3a, acid-loaded twice; the first part represents a control pH_i recovery recorded in HEPES-buffered

solution and the second part, a recovery obtained when tacrine (0.25 mM) was present in the medium. Note that the pH_i changes elicited upon addition or removal of NH_4^+ appeared attenuated compared to control conditions, most likely resulting from the THA-induced increase of β_i. In Figure 4b, the rate of pH_i recovery following acidification (dpH_i/dt), estimated in the absence or presence of tacrine, has been plotted *versus* pH_i; data were obtained from the experiment shown in Figure 4a. It was clear from this figure that the rate of pH_i recovery was markedly slowed down upon exposure to tacrine. However, when constructing the pH_i-dependence of the H^+-equivalent efflux J^e_H (which has been defined as the product of dpH_i/dt and β_i; Roos & Boron, 1981) occurring under both conditions, no difference could any longer be detected (Figure 4c). Figure 5a illustrates the averaged effluxes obtained in the absence or presence of THA ($n=9$ different experiments for control; $n=11$ for THA). As in Figure 4c, a short-term exposure to 0.25 mM THA did not significantly alter the pH_i-dependence of the mean acid efflux occurring after acid loading of RLEC. In contrast, following 24-h treatments with THA, marked alterations of total acid extrusion were seen (Figure 5b). So, whereas this extrusion was significantly reduced in cells exposed to 0.05 mM THA (decrease by $\approx$36% at pH_i 6.73 compared to acid extrusion estimated under control conditions; $P<0.05$), a large increase was detected with a THA concentration of 0.25 mM (becoming significant below a pH_i of 7.05) and no change with 0.10 mM THA.

In Na^+-free medium The observation that THA elicited an alkaline shift of basal pH_i (see Figure 1) suggested that THA might have up-regulated the Na^+/H^+ exchange activity in RLEC. With the aim of testing this hypothesis, a Na^+-free HEPES-buffered solution was used in order to inhibit Na^+/H^+ exchange. As expected (Figure 6a), the cells underwent an immediate intracellular acidification upon Na^+-removal. Under such conditions, addition of THA (0.25 mM) still elicited an intracellular alkalinization; the amplitude of this change was surprisingly significantly enhanced compared to control conditions ($\Delta pH=0.22\pm0.02$ units, $n=6$ [0 Na^+] *versus* 0.16 ± 0.01 units, $n=14$ [control]; $P<0.006$). Furthermore, the rate of the following re-acidification was markedly slowed down, if not totally inhibited (rate $=0.011\pm0.004$ pH_i units min^{-1}, $n=6$ [0 Na^+] *versus* 0.032 ± 0.004 pH_i units min^{-1}, $n=11$ [control]; $P<0.001$). These results showed that the THA-induced alkalinization was not related to Na^+/H^+ exchange activity. The subsequent acidification upon THA-exposure might result either from inhibition of this exchange (passive H^+ influx and acid production then overwhelming pH_i regulation) or from activation of a Na^+-dependent acid loader. Since in the presence of EIPA (50 μM), changes of pH_i upon THA exposure were found to be close to those observed in Na^+-free medium (data not shown), we might suppose an inhibitory effect of THA on Na^+/H^+ exchange. In this context and having demonstrated that, in RLEC superfused with HEPES, the pH_i-recovery following an acid load was brought about essentially *via* Na^+/H^+ exchange (Figure 3), the fact that the total acid efflux remained unaffected by a short-term exposure to THA (see Figure 4c) seemed rather paradoxical. This led us to seek whether, upon THA-exposure, a transport

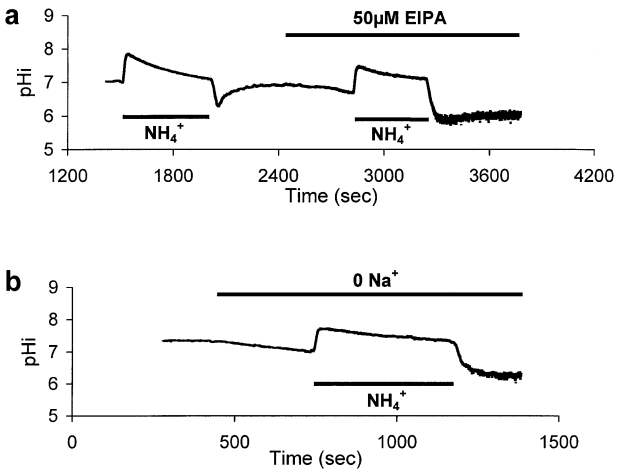

Figure 3 Evidence of Na^+/H^+ exchange activity in RLEC. (a) Effects of the Na^+/H^+ exchange inhibitor EIPA on pH_i recovery from an acid load in HEPES-buffered solution. Addition and removal of 20 mM NH_4Cl (NH_4^+) was used to induce an intracellular acid load. pH_i changes during two different pulses are shown: first pulse, under control conditions; second pulse, in the presence of 50 μM EIPA. No difference was observed between two consecutive pH_i recoveries under control conditions. The trace is representative of four similar experiments. (b) Effects of external Na^+ removal (0 Na^+) on pH_i recovery from an acid load induced by pulse withdrawal of 20 mM NH_4Cl. Data are representative of four similar experiments.

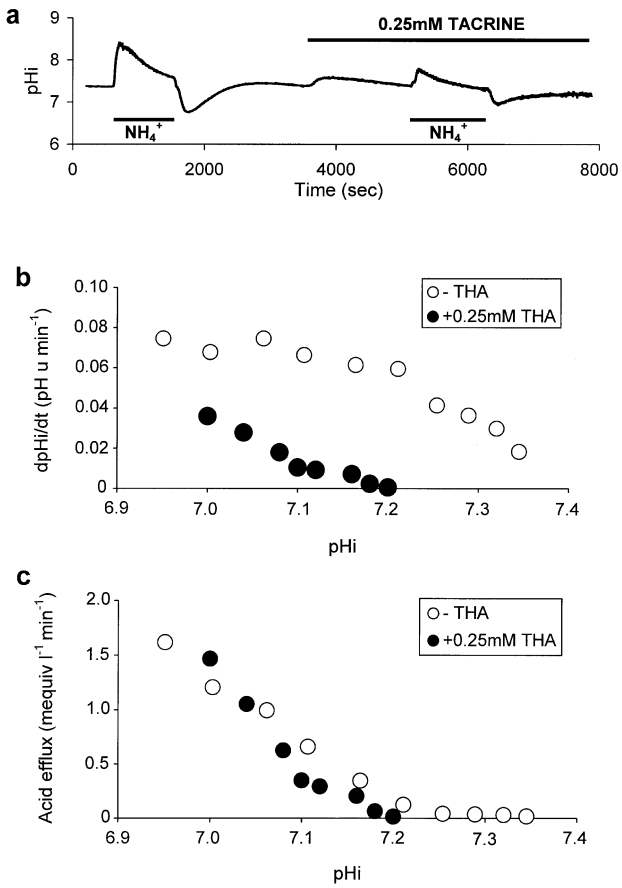

Figure 4 Effects of a short-term application of tacrine (THA, 0.25 mM) on pH_i recovery from an intracellular acidification in HEPES-buffered medium. (a) Typical pH_i recording. RLEC were acid-loaded twice by the NH_4^+ prepulse method: first, in the absence (control recovery) and then in the presence of tacrine. (b) Effects of THA on the pH_i-dependence of the rate of pH_i recovery (dpH_i/dt; u: unit) following an acid load (+0.25 mM THA). pH_i recoveries recorded in (a) were used to calculate dpH_i/dt. (−THA): rate of pH_i recovery in the absence of THA. (c) Representative pH_i dependence of the acid efflux estimated as the product of dpH_i/dt and β_i in the absence or presence of THA (mequiv: milliequivalent). The effluxes were calculated using dpH_i/dt determined in (b).

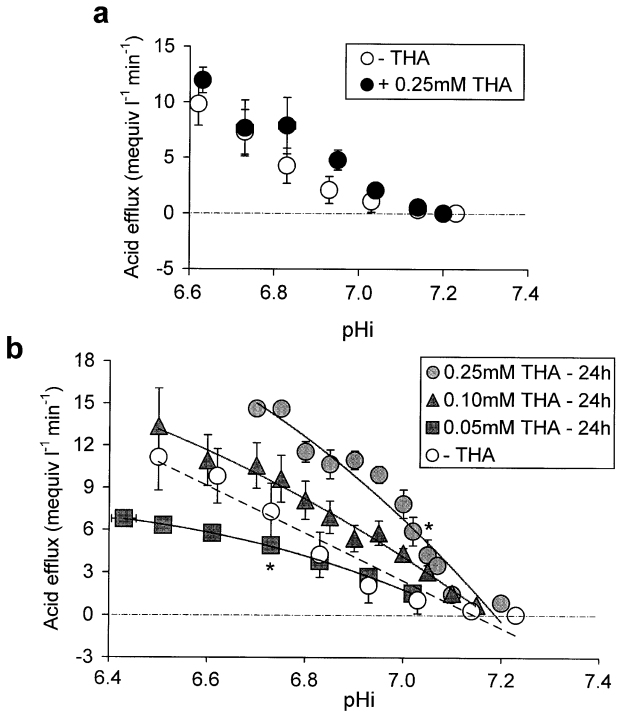

Figure 5 Averaged effects of tacrine on the pH_i dependence of the acid efflux estimated in RLEC bathed with HEPES-buffered solution. Each point represents the average efflux (±s.e.mean) calculated at different test pH_i. (a) Effect of a short-term application of THA (0.25 mM). Data were obtained from nine and 11 separate experiments in the absence (−THA) and presence (+0.25 mM THA) of THA, respectively; mequiv: milliequivalent. (b) Effect of a long-term (24 h) application of THA at different concentrations. Acid efflux was calculated from six, six and five separate cultures treated with 0.05 (0.05 mM THA−24 h), 0.10 (0.10 mM THA−24 h) and 0.25 mM THA (0.25 mM THA−24 h), respectively. Acid effluxes were fitted by polynomial equations in order to ease comparison. The pH_i-dependence of acid efflux calculated in the absence of THA (−THA; *c.f.* a) has been plotted as control. To avoid any further overload of the graph, significant difference ($P<0.05$; treated *versus* control cells, *t*-test) is given only for the pH_i value below which efflux in treated cells becomes different from control.

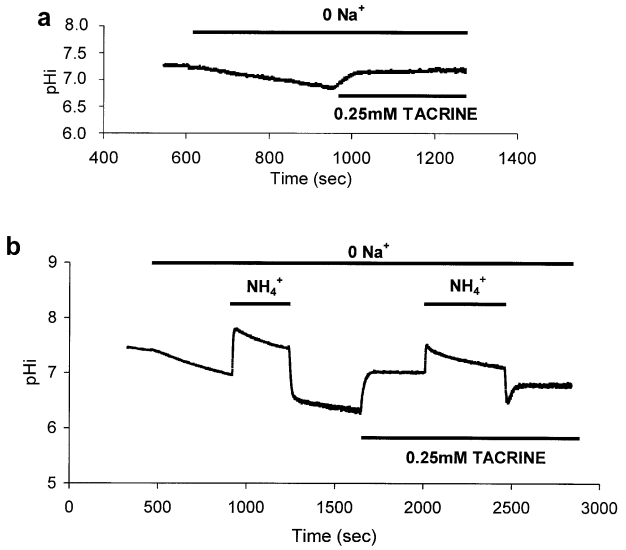

Figure 6 Effects of extracellular Na^+ removal on the effects of tacrine in RLEC. (a) Effects of a short-term application of tacrine (0.25 mM) on basal pH_i recorded in the absence of extracellular Na^+ (0 Na^+). (b) Effects of tacrine on pH_i recovery from an acid load elicited in the absence of extracellular Na^+. RLEC were acid-loaded twice in Na^+-free medium using the NH_4^+-pre-pulse method: first, in the absence and then in the presence of tacrine. Note that in Na^+-free medium, THA induces a pH_i recovery.

mechanism other than Na^+/H^+ exchange could be activated. In Figure 6b, the cells were acid-loaded twice in Na^+-free medium. Although, as expected, pH_i-recovery following the first pre-pulse was totally inhibited under such conditions, a significant rapid pH_i recovery following the second pre-pulse was observed when THA was present. Similar effects were observed with EIPA (data not shown). Figure 7a illustrates the pH_i-dependence of the Na^+-independent acid efflux activated by a short term exposure to tacrine (0.25 mM). While negligible above pH_i 6.9, the contribution of this efflux (J^e_H determined in Na^+-free solution) to total acid efflux (J^{Tot}, estimated in Na^+ containing medium plus tacrine; and equal to the sum of effluxes occurring under such conditions, that is Na^+/H^+ exchange plus Na^+-independent acid efflux) increased with decreasing pH_i (contribution reaching ≈40% of J^{Tot} at pH_i 6.75; at this test pH_i, estimated J^e_H was 4.0 mequiv l^{-1} min^{-1} in THA-containing, Na^+-free solution *versus* 9.8 mequiv l^{-1} min^{-1} in THA and Na^+-containing solution; effluxes were calculated using equations given in the legend of Figure 7a). When estimating $J^{Na+/H+}$ in the presence of THA (by subtracting the acid efflux determined in the absence of extracellular sodium from J^{Tot}, both obtained in presence of THA), it appeared that the acid efflux carried by Na^+/H^+ exchange would be markedly reduced by tacrine (reaching an estimated value of ≈5.8 mequiv l^{-1} min^{-1} at pH_i 6.75 compared to ≈7.3 mequiv l^{-1} min^{-1} under control conditions, i.e. in the absence of THA; see insert in Figure 7a). A Na^+-independent acid efflux was also evidenced following long-term exposures (24 h) to tacrine; its activation being enhanced when increasing THA concentration (Figure 7b). Thus, the set-point of this activation was found to be at pH_i 7.05, 7.0 and 6.7 in 0.25, 0.10 and 0.05 mM THA-treated cells, respectively. Moreover, the contribution of the Na^+-independent acid efflux to total extrusion increased with THA concentration, amounting to about 64 and 80% of J^{Tot} (estimated in the presence of both THA and extracellular Na^+; see Figure 5b) at a pH_i of 6.75, in the presence of 0.10 and 0.25 mM THA, respectively.

Possible activation of a lactate-proton cotransport by tacrine

In order to get further insight into the Na^+-independent mechanism activated by THA, the effects of two known inhibitors of the $Lactate^-$-H^+ cotransport were investigated in Na^+-free medium. Figure 8a shows that the Na^+-independent pH_i recovery activated by THA was markedly inhibited by 4 mM α-cyano-4-hydroxy cinnamate (CHC) while no inhibition was observed in the presence of 0.125 mM quercetin (Figure 8b). Therefore, our results suggest that a quercetin-insensitive, CHC-inhibitable proton-extruding transport might participate in the pH_i-regulation from an acid load when RLEC are exposed to tacrine.

Discussion

In the present work, we have demonstrated that, in RLEC, tacrine induces alterations not only of basal intracellular pH (pH_i) but also of intracellular intrinsic buffering power (β_i) and pH_i-regulating mechanisms. With respect to the latter point, an interesting observation is that while THA down-regulates

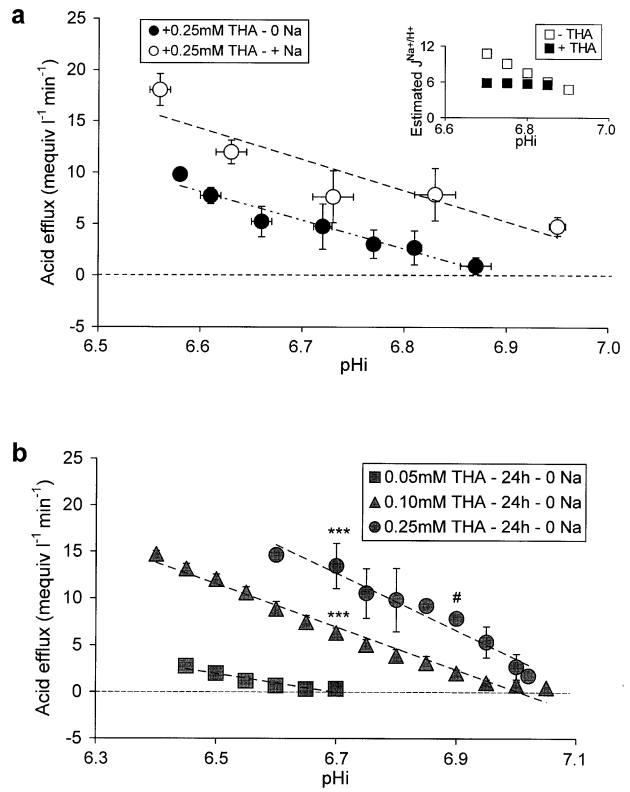

Figure 7 pH_i dependence of the extracellular Na^+-independent acid efflux stimulated by tacrine. (a) Effect of a short-term application of 0.25 mM THA ([+0.25 mM THA−0 Na]; $n=6$ separate experiments; mequiv: milliequivalent). Total acid efflux estimated in THA, Na^+-containing solution (+0.25 mM THA− +Na) has been plotted as indicative. The insert illustrates the acid efflux carried by Na^+/H^+ exchange ($J^{Na+/H+}$) estimated: first, in the absence of THA under control conditions (medium containing Na^+; [−THA]); second, in the presence of THA (+THA), by subtracting the efflux estimated in Na^+-free medium (using the equation: $J^e_H = -27.9 \times pH_i + 192.5$, $r^2=0.94$, $P<0.001$; see main graph) from the one calculated in Na^+-containing solution (using the equation $J^{Tot} = -30.2 \times pH_i + 214$, $r^2=0.84$, $P<0.001$; see main graph). (b) Effects of long-term treatments (24 h) with different concentrations of tacrine on extracellular Na^+-independent acid efflux. $n=5$, six and six separate experiments at 0.05 (0.05 mM THA−0 Na), 0.10 (0.10 mM THA−0 Na) and 0.25 mM THA (0.25 mM THA−0 Na), respectively. ***($P<0.001$; *t*-test): significant difference between the effects at 0.10 or 0.25 mM THA and those at 0.05 mM. #($P<0.02$; *t*-test): significant difference between the effects at 0.10 and those at 0.25 mM THA. The significant level is given only for the pH_i below which the difference appears. The dashed line in (b) represents the control efflux in Na^+-free medium without THA.

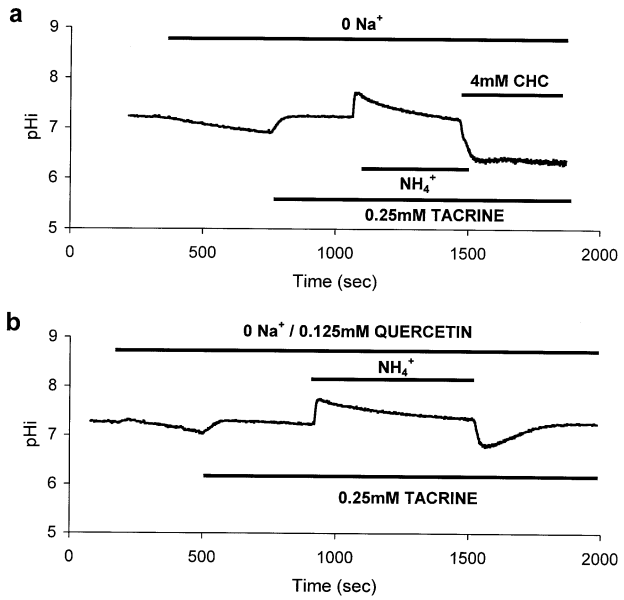

Figure 8 Effects of two different inhibitors of $Lactate^-$-H^+ cotransport on the pH_i recovery stimulated by tacrine in Na^+-free, HEPES-buffered medium (0 Na^+). Cells were acid-loaded by the NH_4^+-prepulse method. (a) Effect of α-cyano-4-hydroxy cinnamate (CHC, 4 mM). The recording was representative of four similar experiments. (b) Effect of quercetin (0.125 mM). The trace was representative of five similar experiments.

Na^+/H^+ exchange, it activates, in a dose-dependent manner, a CHC-inhibitable, Na^+-independent H^+ extruder such that the capacity of THA-treated RLEC to respond to large acid challenges remains high enough to limit any acid load of the cells.

When RLEC were exposed to tacrine in HEPES-buffered medium, a rapid, transient alkalinization was elicited, followed by a phase of acidification. At the lowest concentration used in this study, i.e. 0.05 mM, only the acidification phase could be detected. Several hypotheses might explain the intracellular alkalinization which develops upon tacrine exposure. Based on the literature, one might suppose a stimulation of Na^+/H^+ exchange, which would shift the basal pH_i to a more alkaline value (Strazzabosco & Boyer, 1996). However, under conditions known to inhibit this exchange (i.e. removal of extracellular Na^+ or the presence of EIPA), we found that the THA-induced alkalinization, instead of being inhibited, was enhanced, thus ruling out any role for Na^+/H^+ exchange in the alkaline shift of the basal pH_i observed upon THA-exposure. As an alternative explanation, we might suppose that the THA-induced alkalinization in RLEC was mainly attributable to the properties of this amine. Indeed, as a weak base, only the lipophilic, unprotonated form (T) will cross the sarcolemmal membrane; once inside the cells, tacrine will then get protonated (TH^+). As a consequence, a rise of cytoplasmic pH (pH_i) will occur, the amplitude of which is known to be dependent on extracellular weak base concentration (Roos & Boron, 1981). The properties of tacrine as a weak base may also underlie the increase in intracellular intrinsic buffering power (β_i) evidenced here. Indeed, by reacting directly with exogenous H^+, tacrine may increase the physicochemical buffering of the cell which represents the single most important buffer system (Roos & Boron, 1981). This supposition is reinforced by our data showing that β_i increased with THA concentration. When responding to acid or alkaline loads, cells first recruit several relatively rapid mechanisms (representing β_i) in order to minimize pH_i changes and allow the short-term regulation of pH_i (Roos & Boron, 1981). The fact that β_i was found to be enhanced upon THA exposure might mean that upon such a treatment, RLEC would be able to respond rapidly to any acid challenge (such as a further metabolic acid production that we supposed to occur upon THA treatment considering the simulation of mitochondrial respiration under such conditions; Berson *et al*., 1996) and hence to limit, at least partly, the development of intracellular acidification. The question then arises regarding the fate of long-term pH_i regulation under such conditions. This type of regulation relies upon the activity of several membrane transporters responsible for acid efflux (J^e_H) and allows the cells to recover and maintain their control pH_i.

Another interesting observation from the present work was that the acidification phase which followed the THA-induced alkalinization was markedly, if not totally, inhibited in Na^+-free medium. Therefore, this suggested that, upon tacrine, the

shift of cytoplasmic pH towards acid values might result from an inhibition of Na^+/H^+ exchange. However, activation of a Na^+-dependent acid loader by the THA-induced alkalinization (such as Na^+-HCO_3^- cotransport) cannot as yet be fully ruled out. The down-regulation of Na^+/H^+ exchange by tacrine was actually evidenced when estimating the acid extrusion activated upon intracellular acidification of RLEC. We effectively found that: (i) in cells treated with 0.05 mM THA for 24 h, total acid extrusion was significantly reduced below pH_i 6.75; (ii) following a short-term exposure to 0.25 mM tacrine, the contribution of the THA-activated, Na^+-independent acid efflux to total acid extrusion was estimated to vary from 18–60% in the pH_i range 6.85–6.55, thus pointing to a reduced contribution of the acid efflux carried by Na^+/H^+ exchange (bearing in mind that total acid extrusion represents the sum of all acid effluxes). This slowing of Na^+/H^+ exchange might occur in several ways. Owing to binding of tacrine to membrane acidic phospholipids (Lehtonen *et al*., 1996), cell membrane properties might then undergo modifications. As a result, derangement of intracellular signalling pathways known to control Na^+/H^+ exchange activity (Counillon & Pouysségur, 1995; Noël & Pouysségur, 1995) might occur upon tacrine exposure and thus underlie the slowing of this exchange. We may also suppose an action of tacrine on membrane fluidity which has been shown to affect the activity of Na^+/H^+ exchange (Dudeja *et al*., 1987).

Despite the down-regulation of Na^+/H^+ exchange activity upon tacrine exposure, the total acid efflux occurring after acid loading the RLEC remained surprisingly unaffected, at least following an acute treatment of RLEC with 0.25 mM THA. This observation led us to suppose the activation by tacrine of another proton-extruding process to compensate for the decrease of the Na^+/H^+ exchange. We actually found that in Na^+-free medium or in the presence of EIPA, i.e. under conditions known to inhibit Na^+/H^+ exchange, an H^+ extruder was then functional in THA-exposed cells; this mechanism, which appeared quiescent in the absence of the drug (since during inhibition of Na^+/H^+ exchange, no pH_i recovery occurred) may temporarily help the cells to keep intact their capacity to respond to an acid challenge and hence to prevent, or at least limit, the development of intracellular acidosis simultaneously to the increase of β_i. In support of this idea, it was noted that: (i) following long-term exposure to 0.10 or 0.25 mM THA, steady-state pH_i (which is governed by the balance of several acid-equivalent transporters; Leem *et al*., 1999) was found to be similar to that measured in the absence of THA, despite down-regulation of the Na^+/H^+ exchange; and (ii) activation upon treatment with THA of the Na^+-independent acid extruder increased with THA concentration. In the present study, we have observed that a small, but significant acidification of RLEC at the steady state was elicited only upon a 24-h treatment with 0.05 mM THA and that this effect was related to both a significant decrease of total acid extrusion and a low Na^+-independent acid efflux compared to the other two THA concentrations. Taken together, these results suggest that the development of cell acidification (as observed at 0.05 mM) might result from both a down-regulation of Na^+/H^+ exchange and a low activity of the Na^+-independent acid extruder activated by THA, whereas, at higher concentrations, no such acidification occurred due to a high activity of this latter extruder. Similar to what was presently observed, a compensating process has very recently been described in cardiomyocytes; it concerns the vacuolar H^+-ATPase, the activation of which is involved in the defence of the cells against ischaemia/reperfusion injury, a situation known to affect Na^+/H^+ exchange activity (Karwatowska-Prokopczuk *et al*., 1998). Following the demonstration of a tacrine-activated, Na^+-independent H^+ extruder, further experiments were then carried out in an attempt to characterize this extruder.

In a previous study, we have shown that tacrine affected intermediary metabolism leading to a depletion of the glycogen pools in hepatocytes (Lagadic-Gossmann *et al*., 1998). This might suggest stimulation of glycolysis and hence stimulation of lactate production. Considering a possible effect of tacrine on lactate production, we then supposed that modifications of the transmembrane lactate gradient may help the cells to regulate their pH_i through the activation of a Lactate$^-$-H^+ cotransport, normally quiescent under control conditions. In the course of our experiments, we found that CHC (4 mM), a known inhibitor of Lactate$^-$-H^+ cotransport, totally inhibited the tacrine-activated acid efflux, thus pointing to the involvement of such a cotransport. Such an inhibition was absent when using quercetin (0.125 mM), another inhibitor of Lactate$^-$-H^+ cotransport. These contrasting effects may actually result from the properties of the isoforms of the Lactate$^-$-H^+ cotransport known to show different inhibitor specificities (Poole & Halestrap, 1993; Volk *et al*., 1997). Another explanation might be an effect of CHC on another anion mechanism; in this respect, CHC has been reported to inhibit band 3-mediated inorganic anion exchange in erythrocytes (Halestrap, 1976). Therefore, further work is required to clearly identify the CHC-inhibitable, Na^+-independent H^+ extruder activated by tacrine.

Finally, a major question concerns the *in vivo* relevance of the results reported here since we used THA concentrations (0.05–0.25 mM) higher than those expected *in vivo* at the onset of the hepatic sinusoid (0.05–2.5 μM; Berson *et al*., 1996). We must however emphasize that THA has been demonstrated to accumulate inside the cells (Berson *et al*., 1996), thus leading to higher concentrations than those detected at the hepatic sinusoid. From the present study, it appears that cell exposure to 0.05 mM THA (the lowest concentration used here) resulted in both a slight acid shift of steady-state pH_i and a reduction of total acid extrusion. Therefore, in this context, we might suppose that, should pH_i alterations play a role in THA-induced cytotoxicity, it might be through acidification. Regarding this supposition, it has been described that intracellular acidification could play a deleterious action either directly, by e.g. activating endonucleases or caspases thus leading to apoptosis (Czene *et al*., 1997; Furlong *et al*., 1997), or indirectly, through changes of Ca^{2+} homeostasis (Pierce & Czubryt, 1995). Another important aspect to consider is that in the present work, HEPES was used as extracellular buffer. Therefore, the effects of THA on intracellular pH_i *in vivo* could be different when the HCO_3/CO_2 buffering system is present, since other pH_i-regulating mechanisms (such as Na^+-HCO_3^- cotransport or Cl^-/HCO_3^- exchanger) would then be functional and might be differently affected by THA. Nevertheless, as previously suggested by others, inhibition of Na^+/H^+ exchange (that we have detected upon THA-exposure and that will anyway occur in HCO_3-buffered medium) rather than modifications of steady-state pH_i (governed by the balance of several acid-equivalent transporters; Leem *et al*., 1999) could represent the key point in the early steps of cell death (Benedetti *et al*., 1995b). Indeed, under such conditions, intracellular Na^+ homeostasis might be affected thus leading to deleterious alterations of intracellular Ca^{2+} homeostasis; such a cascade is encountered for example during the ischaemia/reperfusion-induced injury of the heart (Pierce & Czubryt, 1995). Finally, based on the existence of a relationship between Na^+/H^+ exchange activity and bile formation

(Lake *et al*., 1988), the observation that THA affects Na^+/H^+ exchange in RLEC might be predictive of alterations in well differentiated biliary epithelial cells and hence in bile formation upon THA exposure. This remains to be established.

In summary, the present study has demonstrated that tacrine is capable of inducing numerous intracellular pH alterations in liver epithelial cells, such as an increase in the intracellular buffering capacity of these cells and a decrease in Na^+/H^+ exchange activity. Despite this latter effect, we have observed that under such conditions, a CHC inhibitable, Na^+-independent acid extruder then becomes active in these cells, thus limiting the development of any intracellular acidification. Therefore, upon tacrine exposure, this activation might represent an adaptative process.

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