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Time-dependent effects of chloroquine on pH of hepatocyte lysosomes

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Cationic amphiphilic compounds (CACs) are chemicals composed of a hydrophobic aromatic ring(s) and positively charged, hydrophilic side chains [1, 2]. These compounds have high pK values (e.g. 8–10) due to their positively charged side chains and can act as weak bases [2]. Indeed, chloroquine, a prototypic CAC commonly used in studies in cell biology, can accumulate in acidic intracellular compartments, including lysosomes and endosomes; its sequestration in these organelles results in an increase in their pH due to proton consumption [3, 4]. The resultant elevated pH (i.e. weak base action) can cause major alterations in the intracellular trafficking and degradation of macromolecules, affecting receptor-mediated endocytosis, intralysosomal digestion, exocytosis, and the biosynthesis of secretory proteins [5]. Moreover, generalized phospholipidosis (in which the liver is often the major site of phospholipid accumulation) has emerged as a serious adverse effect of the clinical use of some CACs (e.g. amiodarone) [2, 6]. While the exact pathogenesis of the phospholipidosis is obscure, increased intralysosomal pH may play a role.

Since there have been no reports systematically evaluating the time dependency of the weak base actions of chloroquine, we studied the serial *in vivo* effects of chloroquine on the pH of hepatocyte lysosomes.

Methods and Results

Male Sprague–Dawley rats (Harlen Sprague–Dawley, Indianapolis, IN) were fed Purina Laboratory Chow (Ralston Purina Co., St. Louis, MO) and allowed access to water *ad lib*. Chloroquine hydrochloride (Aralen hydrochloride, Winthrop-Breon Laboratories Division of Sterling Drug Inc., New York, NY) was administered intraperitoneally at a dose of 40 mg/kg body weight as chloroquine base. To examine the acute effects of chloroquine, rats were killed at 30 min and at 1, 2, 2.5, 3, 12, 24 and 48 hr after a single injection. To examine the chronic effects of chloroquine, rats were dosed every other day for a total of 4, 6, 8, 10 and 12 days. Control animals received saline injections intraperitoneally using identical dose schedules. Body weights at the start of the experiments were not different between rats in control and treatment groups and ranged from 200 to 245 g. After their respective days of treatment, changes in body and liver weights were not different between control and treatment groups (data not shown).

Intralysosomal pH was measured by flow cytometry in isolated hepatocytes using a modification of a technique described by Murphy [7] for analysis of endocytic vesicles. Fluorescein isothiocyanate dextran (FITC-Dex, mol wt. 70,000; Sigma Chemical Co., St. Louis, MO), a pH-sensitive lysosomotropic fluorescent probe [8], was loaded into hepatocyte lysosomes by intraperitoneal injection 16 hr prior to sacrifice as described [9]. Isolated hepatocytes were prepared from rat liver as previously described [10] yielding approximately 500×10^6 hepatocytes per liver with a viability of greater than 90% by Trypan blue exclusion and a purity of 99% by electron microscopy. Selective sequestration of FITC-Dex in lysosomes was confirmed by subcellular fractionation [9]. Isolated hepatocytes were suspended in Krebs–Ringers–Hepes* solution, and lysosomal pH was measured with a Becton Dickinson FCSD IV flow cytometer (Mountain View, CA) with an excitation wavelength of 488 nm. Emission fluorescence was measured using 530 and 585 nm filters with a 570 nm beam splitter. The 530/585 nm ratio was calculated after counting 20,000 cells. A standard curve for lysosomal pH was prepared by placing the hepatocytes in phosphate-citrate buffers ranging in pH from 4.5 to 6.5. Intralysosomal pH was equilibrated with the buffer pH through the addition of ionophores (10 μM monensin and 10 μM nigericin). Metabolic inhibitors (50 mM 2-deoxyglucose and 50 mM sodium azide) were also added to inhibit proton pump activity. For each point on the standard curve, 20,000 hepatocytes were counted and the 530/585 nm ratio was plotted against buffer pH.

The effect of chloroquine on the pH of lysosomes in isolated hepatocytes is shown in Fig. 1. An immediate chloroquine effect was apparent with pH rising from a

*Hepes, *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid.

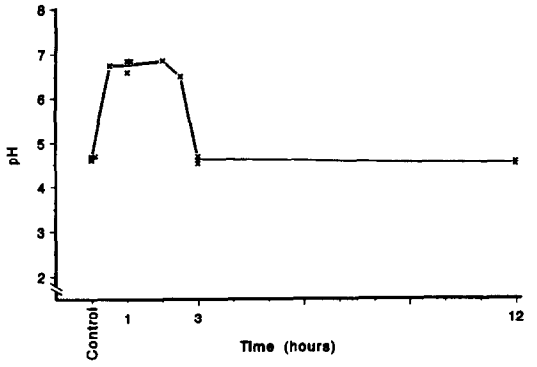

Fig. 1. Effect of chloroquine on intralysosomal pH of isolated hepatocytes. Each point represents an individual pH measurement by flow cytometry as described in Methods and Results. The pH remained unchanged from 3 hr through 12 days.

control pH of 4.65 to 6.75 at the 30-min time point. A peak pH value of 6.85 occurred at 1 hr, followed by a decrease until at 3 hr, the pH returned to baseline and remained unchanged through 12 hr. The pH did not change from baseline at any of the time points in the group of rats treated with the chronic regimen of chloroquine (data not shown).

Discussion

Chloroquine is a typical CAC consisting of a hydrophobic quinoline ring and a positively charged side chain. Owing to two tertiary amines on its side chain, chloroquine can act as diacidic (diprotic) weak base with high pK_a values (10.4 and 8.1) [11]. At physiological pH, the drug assumes the non- or monoprotonated form which is highly permeable to biological membranes [4, 12]. However, upon entering acidic intracellular compartments, including lysosomes and endosomes, chloroquine becomes fully protonated due to the low pH in these organelles; as a consequence, the drug is trapped in these organelles because the diprotonated form cannot permeate cell membranes efficiently [4, 12]. Since the degree of protonation of the drug depends on the acidity of intracellular compartments [13], chloroquine accumulates predominantly in lysosomes, organelles with the lowest pH (approximately 4.8) in cells [5]. Indeed, the intralysosomal concentration of chloroquine has been shown to reach 1000 times that of its extracellular concentration [14].

Our data show a drastic pH increase in hepatocyte lysosomes only at 1 hr after a single dose of chloroquine to intact rats. Ohkuma and Poole [8] reported that the pH of lysosomes increases from 4.8 to 6.4 within minutes of exposure of cultured cells to chloroquine. These *in vitro* results coincide with our own *in vivo* data, although the time-course of the *in vitro* alterations in pH was only partially examined. Reacidification of lysosomes *in vivo* 3 hr after chloroquine administration suggests that the proton pump on the lysosomal membrane in hepatocytes can function quite efficiently to reverse the consumption of intralysosomal protons used for chloroquine protonation.

It has been established previously that CACs increase phospholipid content in liver [2, 6]. For example, a 70% increase in hepatic phospholipid content was reported 12 hr after chloroquine administration [15]. In preliminary experiments, we also observed an increase in total hepatic phospholipid content at 12 hr after chloroquine administration at a time when the pH of hepatic lysosomes had already returned to normal; phospholipid accumulation also occurred at much later time points (i.e. 10–12 days) after chloroquine administration when, as mentioned earlier, the pH of hepatocyte lysosomes was normal [16]. While others have shown that chronic chloroquine can also increase total phospholipid content in liver [17, 18], our results are the first data to document that chronic chloroquine treatment is not associated with alkalinization of hepatocyte lysosomes.

Taken together, these data suggest that the phospholipidosis which occurs after the chronic administration of CACs to animals for experimental purposes or to humans for therapy of certain diseases is not likely to be secondary to CAC-induced changes in pH, since these pH changes are rapid and transient *in vivo*. Indeed, other data suggest that a direct inhibitory effect of CACs, including chloroquine, on hepatic lysosomal phospholipase activity represents the major defect in CAC-induced phospholipidosis [15, 18, 19]. This conclusion is strengthened by our own results indicating that chronic administration of chloroquine does not alter hepatocyte lysosomal pH.

In summary, *in vivo* administration of chloroquine to rats caused an increase in the pH of hepatocyte lysosomes within 1 hr after administration with a return to baseline pH values by 3 hr; continued administration of chloroquine for up to 12 days was unaccompanied by any further changes in hepatocyte lysosomal pH. We interpret these data as evidence against a major role for an increase in the pH of hepatocyte lysosomes in CAC-induced phospholipidosis.

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Acridine orange transport in canine renal brush-border membrane vesicles

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Organic cations are actively secreted by the mammalian kidney [1]. The locus of these transport systems is the proximal tubule. The mechanism of transport across the brush-border membrane is through an organic cation/H^+ antiporter [2]. However, such a mechanism is indistinguishable kinetically and energetically from an organic cation/OH^- symporter. Evidence from several species (rat, rabbit and dog) suggests that this transporter is electroneutral [3–6]. The molecular mechanism of transport may involve a disulfide/sulfhydryl exchange since an essential requirement for disulfide and sulfhydryl groups exists [7]. The H^+ binding site contains essential carboxylate groups [8], whereas the substrate binding site has essential tyrosyl groups [9]. In addition, histidyl groups have been found to be important for transport [10].

Acridine orange, a weak base, has been used to fluorimetrically measure pH changes in brush-border membrane vesicles (BBMV) [11]. Hitherto, its distribution across the plasma membrane had been believed to be solely due to ionic diffusion or nonmediated means. In a previous study, we first observed that acridine orange had an effect on the organic cation/H^+ antiporter in BBMV [12]. The purpose of this report is to clarify this interaction by examining the effect of acridine orange on the transport of N^1-methylnicotinamide (NMN), a prototypic organic cation, in canine renal BBMV. The results demonstrate that acridine orange was transported across the brush-border membrane via the organic cation transporter.

Methods

These studies employed BBMV isolated from the outer cortex of canine kidneys by a divalent cation precipitation [13]. The purified membranes (3.6 to 9.2 mg protein/mL) were suspended in 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), 50 mM K^+ gluconate, 200 mM mannitol, pH 7.5, and were frozen at −70° until used. The pH was adjusted using KOH. All the experiments were done by examining 50 μM [^{3}H]N^1-methylnicotinamide (17.3 Ci/mmol) or 50 μM [^{3}H]*p*-aminohippurate (162 mCi/mmol) transport over a given time period at 37°. The pH of all reaction solutions was 7.5. The assay was initiated by diluting the BBMV 10-fold with the reaction solution (see Figs. 1 and 2). A 100-fold dilution was employed in Figs. 3 and 4 to minimize the carry-over of acridine orange. The details of the experimental procedure have been reported previously [12, 14]. The conditions are outlined in the figure legends. All data are presented as means ± SE. Absence of a standard error bar denotes inclusion within the symbol. Each value was obtained using three to four different membrane preparations performed in quadruplicate. Statistical analysis was performed using ANOVA with testing of the means by the Fisher's test. The radioactive chemicals were purchased from Amersham; all other chemicals came from Sigma.

Results

A concentration–response curve for acridine orange was determined (Fig. 1) and compared to that of verapamil, a competitive inhibitor of the organic cation transport system [15]. The IC_{50} values for these compounds were calculated to be 5.0 and 50 μM respectively. The specificity of acridine orange inhibition was determined by examining what effect, if any, it had on the transport of the prototypic organic anion, *p*-aminohippurate (PAH) (Fig. 2). Acridine orange (20 μM) did not affect PAH transport. The probenecid-inhibitable transport was the same in the presence and absence of acridine orange. This same concentration inhibited NMN transport by greater than 80% (Fig. 1). In