Assessment of Uncoupling by Amiloride Analogs[†]

Kelvin Davies and Marc Solioz*

Department of Clinical Pharmacology, University of Berne, 3010 Berne, Switzerland

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ABSTRACT: The amiloride analogs N^5-methyl-N^5-isobutylamiloride, N^5-ethyl-N^5-isopropylamiloride, and N^5,N^5-hexamethyleneamiloride are frequently used to investigate NaH exchange on the premise that they are highly specific inhibitors of the NaH-antiporters. We assessed the relative protonophoric activity of these compounds in reconstituted and native membrane vesicles, using acridine orange fluorescence to measure intravesicular pH. All the compounds tested were found to be potent protonophores at concentrations which are normally used to demonstrate inhibition of NaH exchange. Uncoupling was dependent on both the pH of the assay system and the total amount of lipid present. At the pH optima, which lay in a range from 7.5 to 8.5, these amiloride analogs were more potent uncouplers than the classical protonophore carbonyl cyanide *m*-chlorophenylhydrazone. Therefore, extreme care must be taken in the interpretation of results obtained using these or similar derivatives of amiloride.

In the study of NaH exchange processes, the diuretic drug amiloride and many of its analogs are widely used as specific inhibitors of NaH antiport. However, these compounds exhibit numerous other effects. They inhibit a broad range of enzymes, including Na^+ channels, NaCa-exchangers, NaK-ATPases, protein kinases, and others [for review see Kleyman and Cragoe (1988) and Benos (1988)]. The parent compound, amiloride, has also been shown to act as an uncoupling agent at concentrations similar to those necessary for the inhibition of NaH exchange, i.e., 0.1–1 mM (Dubinsky & Frizzell, 1983). This property of amiloride could seriously interfere with the interpretation of results derived from experiments investigating NaH exchange.

Today, one generally employs amiloride analogs that are purportedly 100–1000-fold more potent inhibitors of NaH antiport than amiloride (Kleyman & Cragoe, 1988). While there have been cursory mentions of the proportionally stronger uncoupling effect of these compounds (Leblanc et al., 1988; Taglicht et al., 1991), their relative protonophoric activity has never been reported. Indeed, most authors do not acknowledge the possible uncoupling effect of amiloride analogs. Obviously, protonophoric activity of a purported NaH antiport inhibitor will dramatically interfere with experimentation on NaH exchange and the interpretation of the results of such experiments. Therefore, we have investigated the uncoupling activities of some of the commonly used analogs of amiloride, such as the N^5-methyl-N^5-isobutyl and N^5-ethyl-N^5-isopropyl derivatives. We observed that the increased ability of these compounds to inhibit NaH antiport correlates with their increased proton conducting activity. Indeed, under our conditions, they appear more potent than the classical uncoupler CCCP.[1] The ability of the amiloride analogs to act as uncouplers increases with the drug/lipid ratio. The amiloride derivatives express sharp maxima in the uncoupling efficiency between pH 7.5 and 8.5. It has previously been reported that N^5-methyl-N^5-isobutylamiloride specifically inhibits Na uptake by vesicles loaded with NH_4Cl. In light of our results, this observation could be explained not only by an effect on NaH-antiporter activity but also by a dissipation of the proton gradient required to drive this process. Careful control experiments should be performed before conclusions are drawn regarding the mechanism of inhibition by amiloride analogs.

MATERIALS AND METHODS

Chemicals. MIBA and HMA were obtained from Research Biochemicals Inc. (Natick, MA); EIPA was a kind gift from Heinrich Murer, University of Zurich. The amiloride analogs were dissolved in DMSO prior to use. Becton Dickinson (Cockeysville, MD) was the supplier of BBL–trypticase peptone and BBL–yeast extract. CCCP was obtained from Sigma (St. Louis, MO), and stock solutions were prepared in ethanol. Acridine orange was purchased from Fluka AG (Buchs, Switzerland), and *n*-octyl β-D-glucoside was from Bachem Feinchemikalien AG (Bubendorf, Switzerland).

Preparation of Liposomes. The preparation of liposomes was based on the method of Apell and Solioz (1990). Briefly, 40 mg of asolectin (crude soy bean phospholipids) dissolved in ether was dried under vacuum. To the residue was added 2 mL of 10 mM Tris-HCl, pH 7, 0.5 mM dithiothreitol, and 80 mg of *n*-octyl β-D-glucoside, and the mixture was gently shaken until the phospholipid was completely solubilized (approximately 1 h). This solution was first dialyzed for 2 h against 500 mL of the same buffer and then overnight against 500 mL of this buffer containing either 500 mM NH_4Cl or 500 mM NaCl. The resulting suspension of NH_4Cl- or NaCl-loaded liposomes contained about 20 μg/mL lipids.

Acridine Orange Fluorescence Assays. Formation of a pH gradient was followed by the quenching of acridine orange fluorescence at 20 °C as previously described by Rosen (1986). The assay rests on the following principle: acridine orange is a weak base that is membrane permeable in its unprotonated form. Therefore, the distribution of the dye across the membrane will be proportional to the ΔpH. Acridine orange thus accumulates in an acidic vesicle compartment. The resultant high dye concentration leads to self quenching of the fluorescence and a decrease in the overall fluorescence signal.

The reaction buffer for fluorescence measurements consisted of 2 mL of 10 mM Tris-HCl, pH 7.5, 500 mM choline chloride,

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* Address correspondence to this author.

[1] Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; MIBA, N^5-methyl-N^5-isobutylamiloride; EIPA, N^5-ethyl-N^5-isopropylamiloride; HMA, N^5,N^5-hexamethyleneamiloride.

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5 mM $MgCl_2$, and 2 μM acridine orange (buffer A). Quenching was initiated by the addition of 1 μL of the NH_4Cl-loaded liposomes. For uncoupling measurements, liposomes were preincubated with the drugs at the required final concentration for 5 min and then diluted into buffer A containing the drug at the concentration under investigation. To determine the pH dependence of the uncoupling effects of the drugs, NH_4Cl-loaded liposomes preincubated in the presence of the analogs were diluted into buffer A adjusted to the desired pH values with HCl or NaOH.

Fluorescence was measured in a stirred cuvette with a Perkin-Elmer LS50 spectrofluorometer at an excitation wavelength of 494 nm and an emission wavelength of 535 nm. The essentially exponential change in fluorescence following uncoupling was expressed as the exponential "decay" time, τ. For this, the change in fluorescence was replotted on semilogarithmic paper, and from the resultant straight line, we read the time, τ, required for a change in fluorescence from x to x/e. The relative decay rate k was then calculated as $k = 1/\tau$. These rates of dissipation of the proton gradient were usually measured in triplicate for a particular drug concentration, and the values were averaged. The lipid concentration dependence of uncoupling was measured by a similar method, in which varying amounts of vesicles were added to the reaction buffer.

When using sodium-loaded vesicles, the magnitude of the sodium gradient was determined as follows: sodium-loaded liposomes were diluted into sodium-free buffer (buffer A). The stable sodium gradient was subsequently collapsed by the addition of a 50 nM concentration of a sodium/proton-exchanging ionophore (nigericin, monensin), resulting in the generation of a pH gradient. The ensuing acridine fluorescence change allowed precise measurement of the initial sodium gradient.

Preparation of Vesicles from Escherichia coli. E. coli membrane vesicles were prepared from strain W3133-2 (Lopilato et al., 1978) essentially by the method of Rosen (1986). Briefly, cells were grown to stationary phase in 1 L of LB (Maniatis et al., 1989). Cells were harvested by centrifugation and resuspended in a minimal volume of buffer B (10 mM Tris-HCl, pH 7, 0.5 mM DTT, 140 mM choline chloride, 5 mM $MgCl_2$, 250 mM sucrose). The cells were then centrifuged in a preweighed tube and blotted dry with filter paper in order to determine the wet weight. The pellet was suspended in 5–10 volumes of buffer C (as buffer B, but sucrose was replaced by 500 mM NH_4Cl). DNase was added at a concentration of 10 mg/mL and the cells were lysed by passage through a french press at 10 000 psi. The suspension was recentrifuged at 23000*g* for 12 min to remove unbroken cells. The membrane vesicles were then isolated from the supernatant by pelleting the membranes at 100000*g* for 1 h. They were subsequently washed and resuspended in buffer C at a protein concentration of approximately 6 mg/mL. Protein was determined by the Bio-Rad assay, which is based on Coomassie Blue binding (Bradford, 1976). The assay system for the proton gradient developed by these vesicles was the same as described for liposomes. Vesicles were added to buffer A at a final protein concentration of 15 μg/mL, and the decrease in fluorescence was observed when either 20 mM NaCl or 50 μM MIBA was added.

RESULTS

When ammonium chloride-loaded vesicles are diluted into ammonium-free buffer, a pH gradient is generated by the intravesicular dissociation of NH_4^+ into NH_3 and H^+ and subsequent diffusion of NH_3 out of the liposomes. In our experiments, choline chloride was included at a concentration which has been previously shown to collapse a membrane potential in vesicles through the membrane permeability of the chloride ions (Nakamura et al., 1986). Therefore, the quenching of acridine orange is due solely to the pH gradient. The pH gradient generated can conveniently be monitored with the fluorescent dye acridine orange by the method described above (Rosen, 1986). The magnitude of the proton gradient generated (dpH = $pH_{out} - pH_{in}$) can be estimated from $pH_{in} = -\log (NH_{4in}/NH_{4out})(10^{-pK_a} + 10^{-pH_{out}}) - 10^{-pK_a}$, where NH_{4in} is 0.5 M and the pK_a of NH_4^+ is 9.25 (Rottenberg, 1979). Therefore, when buffer A is adjusted to pH 7.5, the calculated pH_{in} is 4.2. However, in reality this value will be somewhat higher due to the intravesicular buffering power.

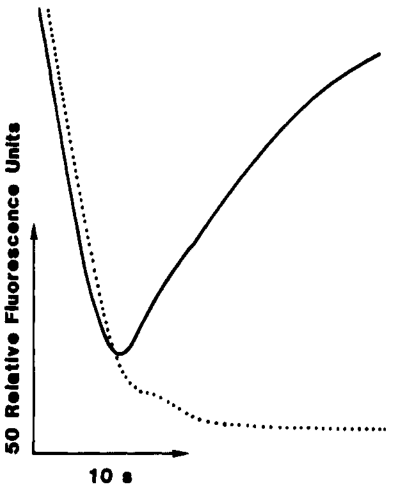

FIGURE 1: Acridine orange fluorescence of ammonium chloride-loaded liposomes. The generation of the proton gradient in liposomes loaded with 0.5 M NH_4Cl and suspended in buffer A, pH 7.5, at 10 μg/mL was measured with control vesicles (dashed line) and vesicles preincubated with 20 μM EIPA (solid line). Details of the experimental conditions are described under Materials and Methods.

Figure 1 shows the fluorescence response from NH_4Cl-loaded vesicles, diluted into NH_4Cl-free buffer. The observed fluorescence quenching (Figure 1, dotted line) was due to the formation of a stable pH gradient, vesicle interior acidic, that persisted for at least 30 min. However, when an amiloride derivative was present, there was only a transient acidification of the intravesicular space (Figure 1, solid line), followed by a rapid loss of protons, as indicated by only transient fluorescence quenching. This protonophoric activity of amiloride analogs was quantified over the active concentration range and for a number of compounds, including the classical uncoupler CCCP. The time constants, k, were then determined from these measurements as described under Materials and Methods. Using sodium-loaded vesicles, we determined that preincubation with amiloride analogs, at concentrations of up to 50 μM, did not result in a detectable loss of sodium from the vesicles.

Figure 2 shows these k values plotted as a function of drug concentration. k increases approximately linearly with increasing drug concentrations. The slope, which is an expression of the relative uncoupling efficiency, exceeds that of CCCP for all of the amiloride analogs shown. Therefore, under these experimental conditions, MIBA, EIPA, and HMA are more potent protonophores than CCCP. N^5,N^5-Dimethylamiloride, while still demonstrating a measurable uncoupling effect, was considerably less effective than the other analogs examined (not shown).

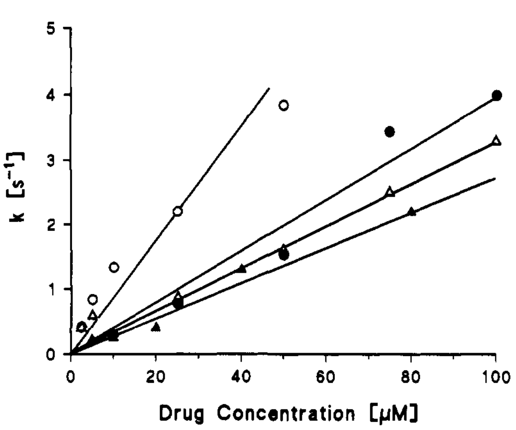

FIGURE 2: Plot of the rate constants, k, versus drug concentration. Uncoupling was measured as described in Figure 1, and the k values were calculated as described under Materials and Methods. Symbols: ○, EIPA; ●, MIBA; △, HMA; ▲, CCCP.

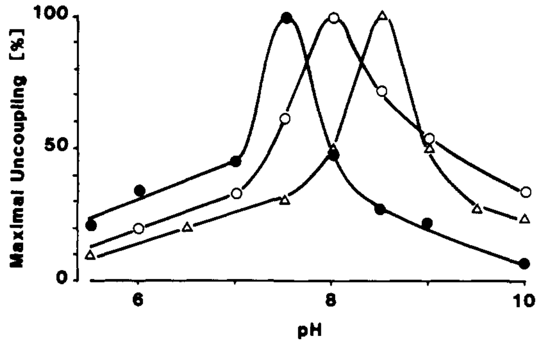

FIGURE 3: pH dependency of uncoupling by various amiloride derivatives. Assays were carried out as described in Figure 1 in buffer A, adjusted to the corresponding pH. Other details are given under Materials and Methods. The following drugs and concentrations were employed: ●, 60 µM HMA; ○, 25 µM EIPA; △, 125 µM MIBA.

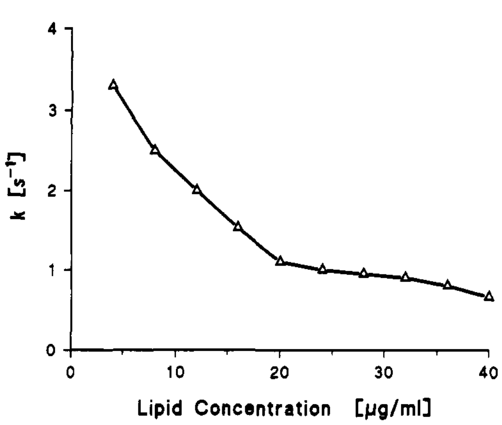

FIGURE 4: Dependence of the uncoupling efficiency on the lipid concentration. Assays were carried out in buffer A, pH 7.5, in the presence of 20 µM EIPA. Other details of the procedure were as described under Materials and Methods.

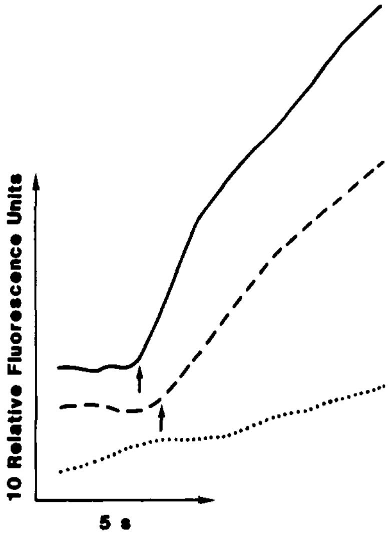

FIGURE 5: Acridine orange fluorescence measurements with *E. coli* vesicles. A 15 µg/mL concentration of *E. coli* vesicles loaded with 0.5 M NH_4Cl was diluted into buffer A, pH 7.5, and the steady-state fluorescence was recorded (dotted line). At the arrow, either 20 mM NaCl (solid line) or 50 µM MIBA (dashed line) was added. One fluorescence unit corresponds to 0.04 pH unit.

At physiological pH, amiloride analogs can be protonated at the guanidino moiety and the 5-amino nitrogen. Since the pK depends on the substituents, it was of interest to look at the pH dependency of the uncoupling effects of the different analogs (Figure 3). The compounds tested all exerted a maximal effect in a narrow pH range, with optima between 7.5 and 8.5. There is no obvious relationship between the pK_a values (reported for HMA and MIBA to be 8.5 and 8.1, respectively; Kleyman & Cragoe, 1988) and the pH optima for uncoupling.

Since the compounds under investigation here are all very hydrophobic in nature, their partitioning between the aqueous phase and the lipid membrane has to be taken into account when looking at concentration-dependent effects. Figure 4 shows the dependency of the uncoupling effect of 20 µM EIPA on the lipid concentration. As the lipid concentration was reduced below 20 µg/mL, the uncoupling effects dramatically increased. Similar results were demonstrable for the other amiloride analogs looked at. Therefore, the ability of these compounds to act as uncouplers critically depends on the experimental conditions in terms of pH and lipid concentration.

To show that these findings with reconstituted liposomes could also be extended to native membrane vesicles, similar experiments were conducted with membrane vesicles of *E. coli*. In Figure 5, ammonium chloride-loaded *E. coli* vesicles were diluted into buffer A, thus developing a stable pH gradient. Upon the addition of sodium there was an increase in the rate of dissipation of the proton gradient, presumably catalyzed by sodium/proton antiport. But the proton gradient could also be rapidly dissipated by the addition of MIBA by means of its uncoupling effect. Similar results were obtained with vesicles prepared from *Enterococcus hirae*.

DISCUSSION

Amiloride analogs dissipated an imposed pH gradient in both native and artificial phospholipid vesicles and thus acted as strong protonophores. The effect was comparable to that of classical uncoupling agents, such as CCCP. Amiloride and the derivatives investigated in this paper are often used in the study of NaH exchange where they are assumed to act as relatively specific inhibitors of NaH-antiporters [e.g., Kleyman and Cragoe (1988), Kapus et al. (1988), Brierly et al. (1989), Desir et al. (1991), and Mochizuki-Oda and Oosawa (1985)]. However, the protonophoric effects of amiloride analogs demonstrated here can be misleading in experiments designed to investigate sodium/proton-exchange processes.

There is a large body of evidence supporting the conclusion that MIBA, EIPA, and HMA are potent inhibitors of NaH-antiporters. While there is little doubt concerning this inhibitory effect, many of the quantitative aspects, such as inhibitory constants, electrogenicity, pH optima, and Na-sensitivity, should be interpreted with great caution in light of our findings.

The protonophoric activity of some amiloride analogs is not always apparent. Dissipating a pH gradient by protonophores requires a charge compensation system, such as valinomycin/potassium. In our experiments, the high Cl^- concentration in the medium paired with the relative leakiness of the membranes for chloride provides the compensatory movement of charges accompanying proton movements. On the other hand, in a tightly coupled system, a pH gradient can be maintained even in the presence of a protonophore and NaH exchange driven by a pH gradient is still apparent (Brierly et al., 1989). The combination of a proton conducting activity by an amiloride analog and a uniporter or a membrane leak for, say, sodium will mimic NaH antiport activity and could introduce serious errors in the interpretation of measurements.

Of the compounds studied here, the most potent NaH-antiport inhibitors are also the most potent uncouplers. In addition, there is a correlation of the potency of the compounds with their hydrophobicity (Kleyman & Cragoe, 1988). This suggests that the efficacy of these analogs, be it as inhibitors or as uncouplers, is increased because of an increase in their partitioning into lipid membranes. Our experiments demonstrating increasing uncoupling with decreasing lipid concentrations clearly illustrate this partitioning effect. For HMA and MIBA, the partitioning coefficient between octanol and 0.1 M phosphate buffer, pH 7.4, was found to be 93 and 94, respectively (Kleyman & Cragoe, 1988), and would be expected to be even higher with lipid membranes as the hydrophobic phase. Thus, the determination of inhibitory concentrations is critically dependent on the lipid concentration employed in the experiments.

We have also demonstrated that the ability of amiloride analogs to act as uncouplers is strongly pH dependent. Many of the variations in K_i values encountered in the literature may thus be ascribed to pH and/or partitioning effects. Observations such as more potent inhibition of growth of *E. coli* by amiloride at alkaline pH (McMorrow et al., 1989) may be explained by more potent uncoupling at higher pH rather than by the need for NaH-antiport activity for growth at alkaline pH.

In conclusion, careful control experiments should be performed before conclusions are drawn regarding the mechanism of inhibition by amiloride analogs. Future experiments will be aimed at determining the partitioning of amiloride analogs into lipid membranes and cellular compartments and the effects of pH and lipid concentrations on these processes. Knowledge of these parameters will hopefully allow more decisive experimentation with these drugs.

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