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Inhibition of Chemotactic Factor-Activated Na^+/H^+ Exchange in Human Neutrophils by Analogues of Amiloride: Structure-Activity Relationships in the Amiloride Series

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SUMMARY

The ability of a number of analogues of the diuretic, amiloride, to inhibit chemotactic factor-stimulated Na^+/H^+ exchange in human neutrophils was investigated. Intracellular pH (pH_i) changes were measured from the equilibrium distribution of ^{14}C-labeled 5,5-dimethyloxazolidine-2,4-dione (DMO). Exposure of cells to 10 nM *N*-formyl-methionyl-leucyl-phenylalanine (FMLP) caused activation of Na^+/H^+ exchange: in 140 mM Na^+ medium (extracellular pH 7.40), the pH_i rose from a resting value of ~7.25 to reach a new steady state of ~7.75 by 10–15 min. This intracellular alkalinization was sensitive to amiloride (apparent K_i ~75 μM), a known inhibitor of Na^+/H^+ countertransport. The structure-activity relationships in the amiloride series were characterized by testing the effect of these compounds on the DMO-derived pH_i changes and on the FMLP-stimulated rate of $^{22}Na^+$ efflux from the cells. Substitutions of the guanidino group of amiloride resulted in relatively inactive products ($K_i \geq 1$ mM). Replacement of the 6-Cl group of amiloride by other halogen atoms had only modest effects on drug efficacy. However, replacement of one or both H atoms of the 5-amino group by short alkyl groups led to a 10–500-fold increase in potency for inhibition of Na^+/H^+ exchange. Amiloride and three of its more potent derivatives (compounds I, O, and MM, the 5-*N*,*N*-dimethyl, 5-*N*,*N*-diethyl, and 5-*N*,*N*-hexamethylene analogues, respectively) caused parallel inhibition of FMLP-activated $^{22}Na^+$ efflux and the rate of intracellular alkalinization, with apparent K_i values of ~75, 8, 1, and 0.2 μM, respectively. In each instance, the inhibitory effects of the drugs were readily reversible on washing the cells. None of the compounds altered the binding of ^3H-labeled FMLP to its cell surface receptors. The development of potent derivatives of amiloride should provide powerful tools for assessing the role of FMLP-activated Na^+/H^+ exchange and the resultant pH_i transients on stimulated neutrophil functions.

The importance of pH_i to a number of intermediary metabolic events and to cellular function is attracting considerable attention. To date, pH_i transients have been shown to play an important role in cell growth and division, contractile properties of muscle, and epithelial transport and secretion (for reviews, see Refs. 1 and 2).

Recently, several workers have proposed that the functional responses of neutrophils, which play a vital role in normal host defense, may be regulated by their pH_i (3–5). These proposals were based, in part, on the original observations of Sha'afi, Naccache, and co-workers (5–8) in rabbit neutrophils and on the subsequent confirmation by our group (9–11), using human neutrophils, that chemotactic factors activate an otherwise quiescent amiloride-sensitive Na^+/H^+ exchange. Under normally prevailing conditions, this cation countertransport system leads to intracellular alkalinization through a 1:1 exchange of internal H^+ for external Na^+.

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The addition of 100 nM FMLP to a suspension of human neutrophils bathed in 140 mM Na^+ medium (pH_o 7.40) leads to dramatic increases in unidirectional $^{22}Na^+$ influxes and effluxes and to a net uptake of Na^+ (9, 10). The intracellular Na^+ concentration rises from 30 to ~60 mM in association with a pH_i transient (i.e., alkalinization) whose time course parallels that of the net gain in internal Na^+, both reaching new steady state values by ~10 min (10, 11). In FMLP-activated cells, the pH_i increases from 7.25 to ~7.80 (11), precisely the increment expected for an electroneutral (i.e., 1:1 stoichiometry) Na^+/H^+ exchange when intrinsic intracellular buffering power [50 mM/pH (12)] is taken into account. All of these net and tracer Na^+ movements, as well as the pH_i changes, were effectively blocked by 1 mM amiloride (10, 11), a well recognized inhibitor of Na^+/H^+ exchange in a variety of cell types (13, 14).

In the present work, we sought to elucidate the structure-activity relationships in the amiloride series with the idea of developing a number of more potent analogues. This effort was pursued for the following reason. In order to block Na^+/H^+

ABBREVIATIONS: pH_i, intracellular pH; FMLP, *N*-formyl-methionyl-leucyl-phenylalanine; DMO, 5,5-dimethyloxazolidine-2,4-dione; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; pH_o, extracellular pH;

exchange in human neutrophils by >90% in our studies (10, 11), we had to use mM concentrations of amiloride. Such relatively high doses of this drug have, to varying degrees, been shown to inhibit Na^+ permeability (15), Na^+/Ca^{2+} exchange (16), and Na^+/K^+ pump activity (17) in other cells. In human neutrophils, we have found that 1 mM amiloride has no effect on passive electrodiffusional $^{22}Na^+$ fluxes through ion channels (10) or on active Na^+/K^+ pump-mediated $^{22}Na^+$ efflux.[1] It is still quite possible, however, that the high concentrations of amiloride used might have other biochemical consequences unrelated to Na^+/H^+ exchange that could perhaps explain some of the inhibitory effects of amiloride on stimulated cell function. The identification of chemical derivatives of amiloride with potencies 2–3 orders of magnitude greater than that of the parent molecule could conceivably prevent these difficulties by increasing specificity.

The idea of developing more potent analogues of amiloride is not new, and several groups have provided a similar rationale for their work in other biological systems. A number of reports have appeared recently in which the structure-activity relationships of various amiloride derivatives have been studied in myocytes (18), fibroblasts (19), and epidermoid carcinoma cells (20). The important general conclusions were that 5-alkylamino-substituted derivatives of amiloride demonstrated substantial increases in potency for inhibition of Na^+/H^+ exchange. The present series of studies was undertaken with two purposes in mind. First, we were interested in clarifying whether similar structural features were important in conferring activity against the Na^+/H^+ exchanger of human neutrophils, a different cell type. The finding of common characteristics in such diverse cell types would thereby serve to imply that important structural elements of the amiloride molecule are conserved. Second, several recent articles have documented that pH_i changes play an important role in the functional behavior of neutrophils. These stimulus-induced responses include phagocytosis, degranulation, superoxide radical generation, and chemotaxis (3, 4, 21, 22). In the last of these reports (22), we observed that an intracellular alkalinization arising from the action of a chemotactic factor-activated Na^+/H^+ exchange mechanism facilitates the directed migration of these cells in a chemotactic gradient. Moreover, inhibition of this pH_i transient by a few selected amiloride analogues abrogated the functional response. It is clear from current trends in the literature that these compounds will, in all likelihood, be used with increasing frequency in the future to probe the effects of pH_i in neutrophils and other phagocytic cells. Thus, in the extensive body of work presented herein, we sought to provide detailed background information for the systematic use of these compounds to study the pH_i-dependent functions of these cells.

In testing more than 50 different compounds, we find that substitutions of the guanidino group of amiloride resulted in relatively inactive products, whereas replacement of the 6-Cl group of amiloride by other halogen atoms had only modest effects on drug efficacy. However, substitutions of one or both H atoms of the 5-amino group of amiloride (apparent K_i ~75 μM) by alkyl groups of increasing length resulted in a 10–500-fold increase in inhibitory potency for Na^+/H^+ exchange.

Methods

Incubation medium. The standard medium used throughout this study had the following composition: 140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5.6 mM glucose, 5 mM HEPES buffer, pH 7.40, and 1 mg/ml of crystalline bovine serum albumin.

Cell isolation. Neutrophils were isolated from heparinized blood of normal donors by the standard procedure of dextran sedimentation at 37° followed by Ficoll-Hypaque gradient centrifugation at room temperature (23, 24). The cellular pellet was resuspended for 30 sec in distilled water to lyse any red cells present. Isotonicity was then restored by addition of NaCl solution, after which the cells were washed twice in standard medium (pH_o 7.40). The cells were kept in this medium for 1 hr at 37° prior to experimentation. About 97% of the cell suspension consisted of neutrophils. Vital dyes, either eosin Y or trypan blue, when added to the suspension, were excluded by more than 99% of the cells, a generally accepted criterion of viability.

Reagents. The following chemicals were purchased from Sigma Chemical Co., St. Louis, MO: DMO, ouabain, HEPES, crystalline bovine serum albumin, and FMLP. FMLP was dissolved at 1 mM in dimethyl sulfoxide (Fisher Scientific, St. Louis, MO) and then diluted in medium. The resultant final concentration of dimethyl sulfoxide (0.001%) had no detectable effect on any of the assays. The isotopes $[^3H]H_2O$, [^{14}C]DMO, [^{14}C]inulin, and ^{3}H-labeled FMLP (specific activity 48 Ci/mmol) were purchased from New England Nuclear, Boston, MA. Amiloride and its analogues were synthesized as previously described (25–29). Cells were pretreated with these compounds for 2 min before experimentation; longer preincubations with the drugs were without further effect.

pH_i Measurements with DMO. All experiments were conducted at 37°. We derived pH_i from the steady state distribution of the ^{14}C-labeled weak acid DMO [pK_a' 6.13 (30)]. In a previous report on pH_i regulation in human neutrophils (12), we had ascertained that DMO-derived pH_i is a convenient measure of *cytoplasmic* pH: the contribution of the strongly acidic lysosomal subcompartment to average pH_i determinations using DMO can be discounted as negligibly small (~0.02 unit). The DMO method has been recently reviewed (1) and will be commented on only briefly. The charged form of this compound is assumed to be impermeant, so that, at steady state, the uncharged partner is equilibrated across the plasma membrane. At known pH_o, pH_i can then be derived from the equilibrium distribution of the indicator. At the shortest practical exposure time, 15 sec, equilibrium of DMO has already been achieved, as evidenced by unchanged distribution for the following 60 min (12). Thus, the probe is suitable for kinetic analyses during which pH_i is changing rapidly.

Samples of the neutrophil suspension (8–12 × 10^6 cells/ml) containing [^{14}C]DMO (1.0 μCi/ml) were incubated in plastic tubes at 37° under various experimental conditions. Unlabeled indicator was added to a total concentration of 0.1 mM, which does not affect pH_i as assessed by the fluorescence of 6-carboxyfluorescein, another pH_i probe (12). At intervals, triplicate aliquots of the cell suspensions were layered over 0.7 ml of Versilube F50 oil (Harwick Chemical Corp., Akron, OH) in 1.5-ml plastic tubes and centrifuged for about 30 sec at 8000 × *g* in a microcentrifuge (Beckman Instruments, Palo Alto, CA). The cells penetrate the oil and accumulate as a pellet, whereas the suspending medium remains above the oil. This layering method, introduced by Naccache *et al.* (6), allows cell separation in less than 5 sec. The pellets were isolated and counted in a liquid scintillation counter (Beckman LS 7000) after addition of 10 ml of Aquasol-2 (New England Nuclear). In preliminary studies, [^{14}C]inulin was added as a marker for the extracellular space. The indicator content of the cells could thus be corrected for the medium trapped in the pellet. The extracellular water was 9 ± 2% (n = 7) of total pellet water (12). In these inulin studies as well as in all others, total water was measured with $[^3H]H_2O$.

We have found (10, 11) that, upon addition of FMLP to a suspension of human neutrophils, only about 60% of the total number of cells actually exhibit a stimulation of $^{22}Na^+$ fluxes (see below). With respect to $^{22}Na^+$ fluxes, the remainder of the cells (~40%) behave as nonresponders. Since the enhanced $^{22}Na^+$ influx and efflux observed following exposure to FMLP may be solely ascribed to transport via Na^+/H^+ exchange (10, 11), it follows that only 60% of the cells will respond

[1] L. Simchowitz, unpublished observations.

with a pH_i change. This conclusion, that $^{22}Na^+$ fluxes and pH_i transients mirror the same transport process, is based on the quantitative (1:1) relationship between the two, as well as on their parallel and complete sensitivity to amiloride (10, 11). In our laboratory, the fraction of FMLP responders (58.7 ± 2.6%, $n = 6$) is similar to the value of 65.0 ± 5.0% ($n = 7$) reported by Seligmann *et al.* (31) who observed functional heterogeneity to FMLP in human neutrophils by means of flow cytometry using a voltage-senstive cyanine dye. Taking into account the existence of discrete neutrophil subpopulations, the DMO-derived pH_i in the bulk cell suspension, $\overline{pH}_{DMO}$, was used to calculate the cytoplasmic pH_i in the FMLP-responding subpopulation, pH_i^{FMLP}, according to the expression (1):

$$\overline{pH}_{DMO} = \log \sum_{n=1}^{j} f_j \cdot 10^{pH_j}$$

where f_j is the fractional volume of the jth compartment. Assuming a two-compartment model (i.e., FMLP-responding cells and non-responders), the equation simplifies to:

$$\overline{pH}_{DMO} = \log\left[f_1 \cdot 10^{pH_i^{FMLP}} + f_2 \cdot 10^{pH_i^{resting}}\right]$$

where the fractional volumes of the FMLP responder and non-responder subpopulations, f_1 and f_2, were taken as 0.6 and 0.4, respectively; the pH_i of the non-responders was taken as the control or resting pH_i in the absence of FMLP. [In preliminary experiments, this assumption was validated by our finding that, using neutrophils loaded with 6-carboxyfluorescein, a fluorescent pH indicator (12), fluorescence microscopy demonstrated uniform, homogeneous cytoplasmic fluorescence throughout the entire resting cell population. Thus, there is no evidence to suggest cytosolic pH_i inhomogeneity in unstimulated cells]. In the figures and tables to follow, all reported pH_i values (and $^{22}Na^+$ efflux rate coefficients) obtained with FMLP have been corrected for the presence of subpopulations, by assuming 60% responding cells.

Unidirectional $^{22}Na^+$ efflux studies. Neutrophils were first suspended at 2–4 × 10^7/ml in standard medium and incubated with $^{22}NaCl$ (5.0 μCi/ml) for 1–2 hr at 37°. The cells were then washed twice, resuspended in unlabeled medium, and treated with 0.1 mM ouabain to inhibit the Na^+/K^+ pump (for rationale, see Results). The incubations, which were performed at 37° in capped, plastic tubes (Falcon Plastics, Oxnard, CA) in a total volume of 1.8 ml containing 12–18 × 10^6 cells, were begun by addition of the stimulus (10 nM FMLP) at zero time. At stated intervals, triplicate 0.5-ml aliquots were layered on 0.7 ml of silicone oil and centrifuged as described above. The neutrophil pellets were excised and counted in a gamma counter. In order to correct for the daily variation in the extent of cell loading, the data were normalized to 1.0 at zero time for each experiment by dividing the cpm at any time by the number of cpm present in the cell pellet at zero time.

^3H-labeled FMLP binding assays. Binding studies were performed at 37° in the presence of 10 nM ^3H-labeled FMLP. At stated times, aliquots of the neutrophil suspensions were layered over silicone oil and handled as described above. The cell pellets were counted for radioactivity in order to determine total binding. Nonspecific binding, defined as those counts "bound" in the presence of a 1000-fold excess of unlabeled FMLP, was ~10% of total binding. The results are expressed in terms of specific binding (i.e., total minus nonspecific binding).

Data analysis. In many instances, the time course of pH_i following the addition of FMLP to the cell suspension could be described by a single exponential equation of the form:

$$pH_t = pH_\infty - [pH_\infty - pH_{initial}]\exp(-kt) \quad (1)$$

where $pH_{initial}$, pH_t, and pH_∞ are the pH_i values at, respectively, zero time, time t, and after steady state had been reached, and k is the rate coefficient. Curves representing the equation were fitted to the various groups of data by the least squares method. The initial rate of pH_i change was calculated from the expression $k[pH_\infty - pH_{initial}]$. In other cases, the pH_i course was nearly linear over the period of study, and the slope of the linear regression represented the rate of pH_i change.

In unstimulated cells, $^{22}Na^+$ effluxes followed single exponentials of the form:

$$C_t = C_0\exp(-kt) \quad (2)$$

where C_t and C_0 are the relative cell labels at, respectively, time t and zero time. The rate coefficient (k) was determined by least squares fitting the data to Eq. 2. In a few instances, as in the analysis of FMLP-stimulated $^{22}Na^+$ efflux (see text), the data were fit to a least squares program as the sum of two falling single exponentials of the form:

$$C_t = C_1\exp(-k_1t) + C_2\exp(-k_2t) \quad (3)$$

where C_1 and C_2 correspond to the cell labels at zero time in compartments 1 and 2 (i.e., FMLP responder and non-responder subpopulations of cells, respectively), and k_1 and k_2 are their respective rate coefficients. In this program, the underlying assumptions are as follows: 1) the sum of the initial magnitudes of the two compartments, C_1 and C_2 (FMLP responders and non-responders, respectively), equals 1.0; 2) both compartments extrapolate to zero at infinite time [i.e., all cell $^{22}Na^+$ is exchangeable (24)]; and 3) k_1, the rate coefficient of the FMLP responders, is refined, whereas k_2, the rate coefficient of the non-responders, equals the rate coefficient of the control cells (i.e., k of Eq. 2).

Results

In agreement with the findings of Sha'afi and co-workers in rabbit neutrophils (6, 8), our group has reported that FMLP stimulates bidirectional $^{22}Na^+$ fluxes in isolated human neutrophils (9, 10). The enhanced rates of $^{22}Na^+$ influx and efflux across the cell membrane represent transport through an amiloride-sensitive Na^+/H^+ exchange that is activated by the chemotactic factor. We have also shown that unidirectional $^{22}Na^+$ influxes and effluxes are competitively inhibited by amiloride with similar apparent K_i values (~75 μM). For technical convenience, in order to facilitate the screening and testing of a large number of amiloride analogues, we chose to measure the effects of these drugs on FMLP-induced $^{22}Na^+$ efflux. All experiments were performed in the presence of 0.1 mM ouabain, which abolishes active $^{22}Na^+$ efflux by the ATP-driven Na^+/K^+ pump (24). This pump-mediated flux constitutes ≥95% of total unidirectional $^{22}Na^+$ efflux from resting cells (24) and would otherwise obscure the true magnitude of the FMLP-activated increment in $^{22}Na^+$ efflux. It should be noted that we have also demonstrated a lack of effect of ouabain on FMLP-induced $^{22}Na^+$ influx (10) or on the kinetics of the FMLP-stimulated intracellular alkalinization (11).

The time course of FMLP-induced $^{22}Na^+$ efflux from cells bathed in 140 mM Na^+ medium (pH_o 7.40) is shown in Fig. 1. The kinetics followed a single exponential (rate coefficient 0.127 ± 0.016 min^{-1}) with the loss of cell $^{22}Na^+$ reaching a plateau by ~30 min, by which time the cells had lost roughly 60% of their total $^{22}Na^+$ content present at zero time. The appearance of this plateau requires some explanation. As previously examined in detail (10), FMLP stimulation of $^{22}Na^+$ influxes and effluxes is sustained (at least through 1 hr) and does not represent a transient event. Rather, the plateau seen at 20–30 min is due to depletion of the $^{22}Na^+$ label in the roughly 60% of the total cell population that actually responds to FMLP. The remaining label, corresponding to about 40% of the total, is contained in a non-responding subpopulation of neutrophils. When analyzing the effect of different compounds on FMLP-stimulated $^{22}Na^+$ efflux, the respective rate coeffi-

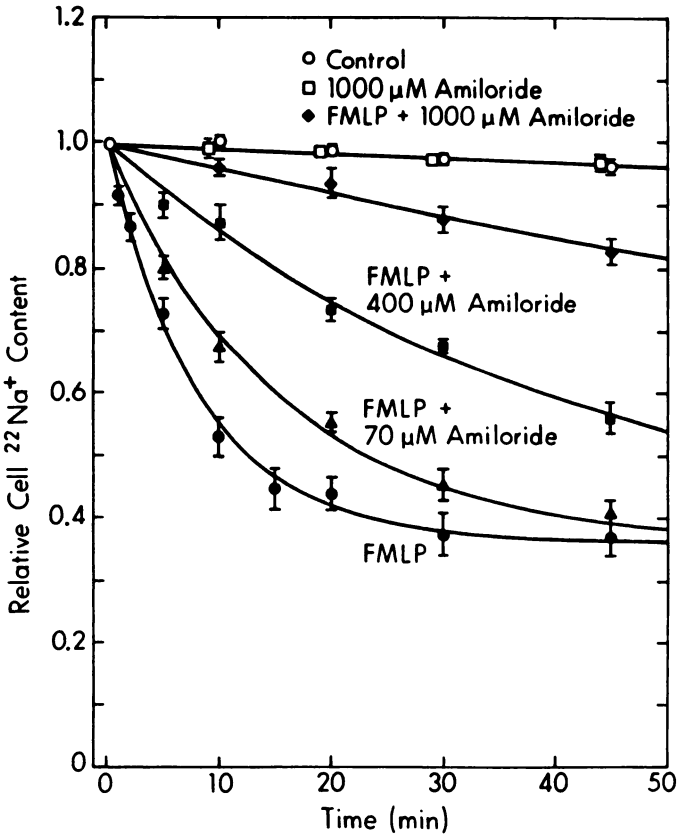


Fig. 1. Time course of FMLP-stimulated $^{22}Na^+$ efflux: effect of amiloride. Experiments were performed in standard medium (140 mM Na^+, pH_o 7.40) containing varying concentrations of amiloride (0–1000 μM). The neutrophils were first loaded with $^{22}Na^+$ for 1–2 hr in the absence of all drugs. The $^{22}Na^+$-labeled cells were then pretreated for 5 min with 0.1 mM ouabain and all experiments were conducted in the presence of the drug in order to inhibit the Na^+/K^+ pump-mediated efflux of the $^{22}Na^+$ label. At zero time, 10 nM FMLP (●, ▲, ■, ♦) was added to the tubes in the presence or absence of amiloride; controls (○, □) were performed in the absence of the stimulus. At stated times, the cell pellets were isolated and the relative cell $^{22}Na^+$ content was determined as described in Methods. Results represent the mean ± standard error of three separate experiments each performed in triplicate. For the combined control (○) and 1000 μM amiloride (□) data (the differences between the two individual sets were insignificant), the data points were fit to a single exponential equation (Eq. 2) by a least squares program: the rate coefficient was 0.00074 ± 0.00012 min^{-1}. Considering the existence of distinct neutrophil subpopulations (see text), the curve labeled *FMLP* was computer fitted to a program as the sum of two falling exponentials (Eq. 3, i.e., that of the FMLP-activated and non-responding subsets of cells). The equation yielded 0.626 ± 0.027 as the fraction of FMLP-responding cells (rate coefficient of $^{22}Na^+$ efflux 0.127 ± 0.016 min^{-1}) and 0.374 ± 0.022 as the fraction of non-responding cells (rate coefficient 0.00074 ± 0.00012 min^{-1}). The rate coefficients of $^{22}Na^+$ efflux from FMLP-activated cells in the presence of 70, 400, and 1000 μM amiloride were 0.0667 ± 0.0040, 0.0249 ± 0.0015, and 0.00625 ± 0.00072 min^{-1}, respectively.

cients in the presence of varying concentrations of each drug were calculated as in Fig. 1 by assuming 60% FMLP-responding cells. It should be pointed out that none of the derivatives of amiloride had any effect on ouabain-insensitive $^{22}Na^+$ efflux in the absence of FMLP, which behaves as passive, electrodiffusional efflux through ion channels (10, 24). Also shown in Fig. 1 is the inhibitory effect of amiloride on FMLP-induced $^{22}Na^+$ efflux from the cells. As the concentration of amiloride was raised from 70 to 1000 μM, there was a progressive decline in the rate coefficient of FMLP-stimulated $^{22}Na^+$ efflux.

In Fig. 2, the dose dependencies for amiloride and for three other analogues (compounds I, O and MM) on the FMLP-activated rate coefficient of $^{22}Na^+$ efflux are displayed. The data points were fitted to Michaelis-Menten inhibition equations which yielded apparent K_i values for amiloride and compounds I, O, and MM of 83.8 ± 16.3, 6.86 ± 1.54, 1.15 ± 0.28, and 0.16 ± 0.04 μM, respectively.

Tables 1–3 list the formulas and apparent K_i values for inhibition of FMLP-activated $^{22}Na^+$ efflux by 41 of the more than 60 different amiloride analogues tested. As will be discussed below, a number of important points concerning the structure-activity relationships in the amiloride series can be made.

The effect on activity of a variety of substituents either on or for a terminal amino group of the guanidino moiety of amiloride was evaluated. The results are summarized in Table 1, where it can be seen that each of the compounds exhibits an apparent K_i value which is at least 10-fold greater than that of amiloride. The substituents include groups such as hydroxy (J), cyano (F), and carbamoyl (E), as well as replacement of the amino group by SH (the thiourea analogue, L). Each of these structural changes is known to reduce the basicity of the molecule (29). Likewise, substitution by an *N,N*-dimethylamidino group (Y) to form the more basic biguanide analogue had a similar detrimental effect on activity. Interestingly, a phenyl (phenamil), benzyl (benzamil), 2-phenethyl (GGG) or tertiary-octyl (FFF) substituent markedly reduced activity. These substituents generally markedly *enhanced* the inhibitory effects on the Na^+ channel (32) and the Na^+/Ca^{2+} exchanger (16) in other cell types as compared to amiloride.

The influence of various 6-position substituents on activity is recorded in Table 2. Replacement of the 6-Cl group of amiloride by 6-H (K) produced a 13-fold reduction in activity.

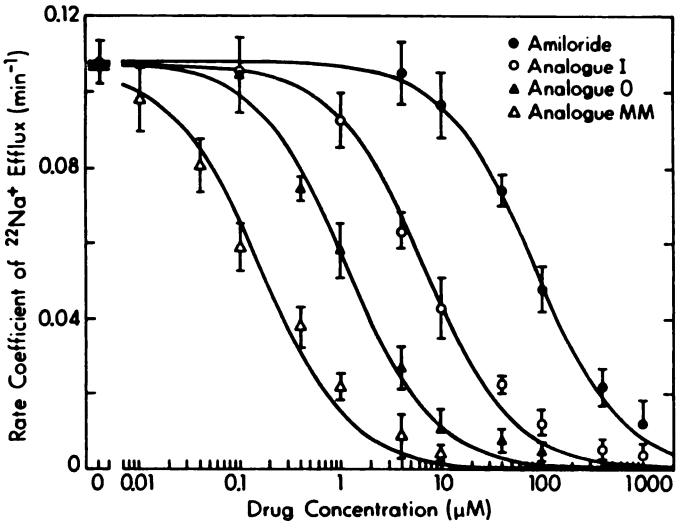
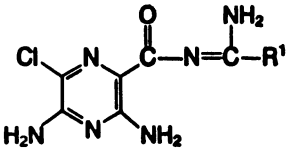


Fig. 2. Concentration dependence of amiloride and several of its analogues on the rate coefficient of $^{22}Na^+$ efflux from FMLP-activated cells. Experiments were performed as in Fig. 1 in the presence of varying concentrations of amiloride or compounds I, O, or MM. For each drug concentration, the rate coefficient of FMLP-stimulated $^{22}Na^+$ efflux was computed on the assumption of 60% FMLP-responding cells. Results represent the mean ± standard error of three experiments. The data points were fit by a least squares program to a Michaelis-Menten inhibition equation which yielded apparent K_i values of 83.8 ± 16.3, 6.86 ± 1.54, 1.15 ± 0.28, and 0.16 ± 0.04 μM, respectively, for amiloride and compounds I, O, and MM.

TABLE 1

Effect of substitutions on the terminal nitrogen atom of the guanidino moiety of amiloride on FMLP-induced $^{22}Na^+$ efflux

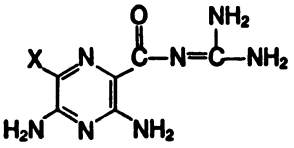
Experiments were performed as in Fig. 1 in the presence of 10 nM FMLP and varying concentrations of each of the designated compounds. The incubations were terminated at 10 min and the rate coefficients of FMLP-activated $^{22}Na^+$ efflux were calculated as in Fig. 1 on the assumption of 60% FMLP-responding cells. The respective rate coefficients were then plotted against the drug concentration. The data points were fitted to a Michaelis-Menten inhibition equation to derive the apparent K_i values as in Fig. 2. Results represent three to four separate experiments.



Code name or letters	R^1	Apparent K_i
		μM
Amiloride	$-NH_2$	83.8
J	$-NHOH$	929
F	$-NHCN$	>1000
L	$-SH$	>1000
E	$-NHCONH_2$	>1000
Y	$-N{=}C(NH_2)N(CH_3)_2$	>1000
Phenamil	$-NH$⬡	>1000
Benzamil	$-NH_2CH_2$⬡	>1000
GGG	$-NH(CH_2)_2$⬡	>1000
FFF	$-NHC(CH_3)_2CH_2C(CH_3)_3$	>1000

TABLE 2

Effect of 6-substituents on the amiloride molecule on FMLP-induced $^{22}Na^+$ efflux[a]

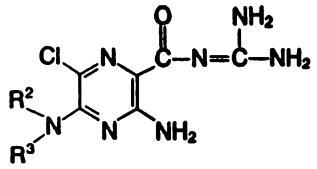


Code letter(s)	X	Apparent K_i
		μM
Amiloride	—Cl	83.8
K	—H	1084.7
D	—F	706.7
Br-AM	—Br	44.2
I-AM	—I	18.3

[a] See legend to Table 1.

TABLE 3

Effect of substituents on the 5-amino group of amiloride on FMLP-induced $^{22}Na^+$ efflux[a]



Code letter(s)	Total C-atoms ($R^2 + R^3$)	R^2	R^3	Apparent K_i
				μM
Amiloride	0	—H	—H	83.8
NN	4	—H	$-(CH_2)_3CH_3$	12.7
DD	4	—H	$-C(CH_3)_3$	3.2
HH	5	—H	$-CH(C_2H_5)_2$	2.9
EE	6	—H	$-(CH_2)_5CH_3$	4.5
TT	3	—H	—◁	13.2
PP	4	—H	$-CH_2$—◁	8.3
JJ	5	—H	—⬠	3.8
KK	3	—H	$-CH_2CH{=}CH_2$	11.4
SS	3	—H	$-CH_2C{\equiv}CH$	10.1
LL	6	—H	—⬡	3.9
N	7	—H	$-CH_2$—⬡	12.7
CCC	7 (+F)	—H	$-CH_2$—⬡	9.7
BBB	8	—H	$-CH_2$—⬡—CH_3	8.7
FF	8	—H	$-(CH_2)_2$—⬡	6.7
XX	5 (+O)	—H	$-CH_2$—◇	10.2
G	1 (+2N)	—H	$-C(=NH)NH_2$	23.4
I	2	$-CH_3$	$-CH_3$	6.9
OO	3	$-CH_3$	$-C_2H_5$	0.63
O	4	$-C_2H_5$	$-C_2H_5$	1.15
QQ	4	$-CH_3$	$-CH(CH_3)_2$	0.87
GG	5	$-CH_3$	$-(CH_2)_3CH_3$	0.24
P	7	$-(CH_2)_2CH_3$	$-(CH_2)_3CH_3$	0.37
VV	4	$-CH_3$	$-CH_2CH{=}CH_2$	0.42
UU	4	$-(CH_2)_4-$		0.68
MM	6	$-(CH_2)_6-$		0.16
S	5 (+N)	$-(CH_2)_2N(CH_3)(CH_2)_2-$		18.5
R	1 (+N)	$-NH_2$	$-CH_3$	9.6
II	2 (+O)	$-OCH_3$	$-CH_3$	6.7

[a] See legend to Table 1.

Replacement by the more hydrophilic 6-F (D) group resulted in about an 8-fold loss in activity. However, replacement by the more lipophilic[2] 6-Br (Br-AM) and 6-I (I-AM) produced, respectively, a 2-fold and 5-fold increase in potency.

The most profound beneficial structrual changes were observed when the 5-amino group of amiloride bore one or two substituents. Among the 16 monosubstituted compounds studied, some structure-activity trends can be noted as summarized in Table 3. Increasing the number of carbon atoms in the alkyl chain was beneficial to activity (NN versus EE) and branched alkyl groups were more effective than straight chains (DD and HH versus NN). Cycloalkyl (TT and JJ) and cycloalkylalkyl (PP) groups appeared to be at least as effective as alkyl groups of the same carbon atom content (PP versus NN and JJ versus HH). Alkenyl (KK) and alkynyl groups (SS) exhibited about the same effects as other groups with the same carbon atom content (TT). A phenyl substituent (LL) was as effective as a carbon atom equivalent alkyl group (EE), but benzyl (N), substituted benzyl (CCC and BBB), 2-phenethyl (FF), and 2-furylmethyl (XX) groups were slightly less activity-enhancing than phenyl. An amidino moiety (G), which imparts a second basic (protonated) center, produces only a small increase in activity.

Compounds bearing two substituents on the 5-amino nitrogen atom were generally more potent than those bearing the same number of carbon atoms in a single substituent (compare

[2] E. J. Cragoe, Jr., unpublished observations.

OO with TT, O and QQ with NN and DD, and GG with HH). Alkenyl groups (VV) were as effective as alkyl groups (QQ). When one substituent was methyl, there was generally an increase in activity as the chain length of the second substituent was increased (I < OO < GG). Joining the two substituents on the 5-amino nitrogen atom to form a ring (UU and MM) has a further enhancing effect on activity; MM, which is 524-fold more active than amiloride, proved to be the most potent compound evaluated in this study. Insertion of a nitrogen atom (bearing a methyl group) into the ring (S) is markedly less beneficial than its carbon isostere. Replacement of one of the methyl groups of I by amino (R) or methoxy (II) had little effect on activity. It should be pointed out that inhibition by several of the more potent derivatives (e.g., compounds O and MM) was readily reversible when, after washing, the cells were exposed to FMLP in the absence of the drugs.

In the next series of experiments, we tested the ability of several of these amiloride analogues to inhibit the intracellular alkalinization elicited by exposure of human neutrophils to FMLP. Since, as indicated by previous work (5–11), these pH_i transients are mediated through an FMLP-activated exchange of external Na^+ for internal H^+, there should be an excellent correlation between the ability of each drug to cause inhibition of FMLP-stimulated $^{22}Na^+$ efflux and that of inhibition of FMLP-induced intracellular alkalinization. These predictions are verified by the data of Figs. 3 and 4. For this purpose, in addition to amiloride, we chose to study a few representative analogues, namely I, O, and MM, whose apparent K_i values for inhibition of FMLP-induced $^{22}Na^+$ efflux differ from that of the parent compound by factors of 10–500.

Fig. 3 shows the kinetics of the DMO-derived pH_i changes elicited by 10 nM FMLP. Following the addition of the stimulus, the pH_i increased from its control value of 7.23. The intracellular alkalinization was evident as early as 15 sec, the earliest time point conveniently measured. Thereafter, the degree of alkalinization increased along an exponential time course (initial rate 0.213 ± 0.025 pH/min) to reach a new steady state pH_i of ~7.75 by 10–15 min. Also displayed in Fig. 3 is the effect of compound MM on the FMLP-induced pH_i changes. As the concentration of analogue MM was raised from 0.2 to 10 μM, there was a progressive reduction in the rate of intracellular alkalinization. However, the final extrapolated pH_i value remained unchanged. This is because, in the presence of FMLP, the final steady state pH_i value is that given by an equilibrium distribution of Na^+ and H^+ across the cell membrane (11). Thus, amiloride and its analogues affect only the rate at which the new pH_i value is reached, not its final extent.

Similar studies were performed using amiloride or derivatives I and O at several different concentrations. The initial rates of FMLP-stimulated alkalinization in the presence of each of the four compounds were compiled in Fig. 4 and plotted against the concentration of each drug. The data points for each analogue were fitted to a Michaelis-Menten inhibition equation which yielded apparent K_i values for amiloride and compounds I, O, and MM of 65.0 ± 11.1, 9.74 ± 1.58, 1.37 ± 0.31 and 0.14 ± 0.02 μM, respectively. These values are in good agreement with those measured for inhibition of FMLP-stimulated $^{22}Na^+$ efflux: 83.8, 6.89, 1.15, and 0.16 μM, respectively (Fig. 2).

Data on the time course of ^3H-labeled FMLP binding to neutrophils in the presence of amiloride or its analogues are given in Fig. 5. As originally reported by Williams *et al.* (33),

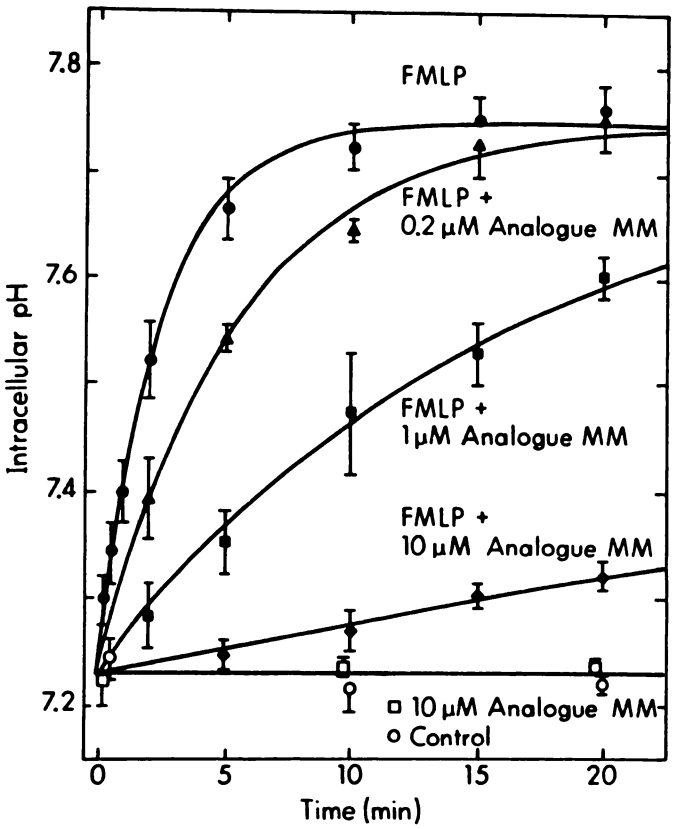


Fig. 3. Time course of intracellular alkalinization in FMLP-activated cells: effect of compound MM. Neutrophils were suspended in standard medium (140 mM Na^+, pH_o 7.40) containing varying concentrations (0–10 μM) of analogue MM in the presence (●, ▲, ■, ♦) or absence (○, □) of 10 nM FMLP. At stated times, aliquots of the cell suspensions were taken for pH_i measurements using [^{14}C]DMO. The DMO-derived pH_i values have been adjusted for the presence of neutrophil subpopulations, by assuming 60% FMLP-responding cells. Results represent the mean ± standard error of three separate experiments, each performed in triplicate. The *top four curves* are single exponential fits (Eq. 2) to the data, where the final extrapolated pH_i value was 7.75 ± 0.02. The initial rates of FMLP-induced alkalinization in the presence of 0, 0.2, 1.0, and 10.0 μM compound MM were 0.213 ± 0.025, 0.0952 ± 0.0094, 0.0313 ± 0.0034, and 0.00496 ± 0.00068 pH/min, respectively. A *horizontal line* was drawn at 7.23, the average of the Control and 10 μM analogue MM data points. The pH_i of unstimulated cells was likewise unaffected by 0.2 or 1.0 μM drug MM. To maintain clarity, these points have been omitted.

the binding of 10 nM FMLP to its plasma membrane receptors on human neutrophils at 37° was rapid (half-time ~2 min), reaching a steady state by ~15 min. Neither the rate nor the final extent of binding was significantly affected by amiloride or compounds I, O, or MM, each at concentrations (10–100-fold greater than their respective apparent K_i values for inhibition of Na^+/H^+ exchange) that markedly inhibited $^{22}Na^+$ efflux and the rate of intracellular alkalinization induced in the cells by FMLP. Nonspecific binding (not shown) was likewise unaltered in the presence of the drugs. Thus, these results provide strong evidence that the inhibition of FMLP-stimulated Na^+/H^+ exchange by amiloride and its derivatives is unrelated to an effect of the drugs on receptor-ligand interactions.

Discussion

Three major structural requirements for activity in inhibiting the Na^+/H^+ exchanger of human neutrophils were examined.

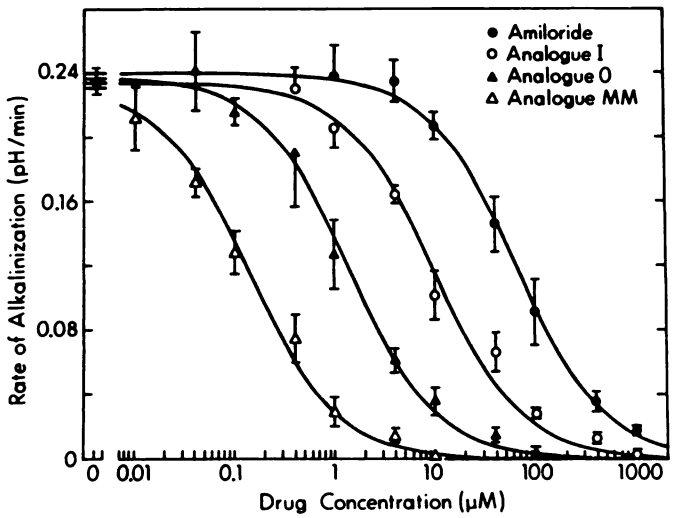


Fig. 4. Effect of amiloride and several of its analogues on the rate of intracellular alkalinzation in FMLP-activated cells. Experiments were performed as in Fig. 3 in standard medium (140 mM Na^+, pH_o 7.40) containing varying concentrations of amiloride or one of several derivatives in the presence or absence of 10 nM FMLP. For each drug concentration, DMO-derived pH_i was measured at two different time points (0.5, 1, 2, 5, or 10 min as appropriate) during the course of the FMLP-stimulated alkalinization. The control (i.e., initial) pH_i and the two measured pH_i values were fitted to a single exponential equation (Eq. 2). The initial rate of pH_i change was calculated from Eq. 2 after adjustment for neutrophil subpopulations by assuming 60% FMLP-responding cells. Results represent the mean ± standard error of three separate experiments, each performed in triplicate. The data points were fit by a least squares program to a Michaelis-Menten inhibition equation which yielded apparent K_i values of 65.0 ± 11.1, 9.74 ± 1.58, 1.37 ± 0.31, and 0.14 ± 0.02 μM, respectively, for amiloride and compounds I, O, and MM.

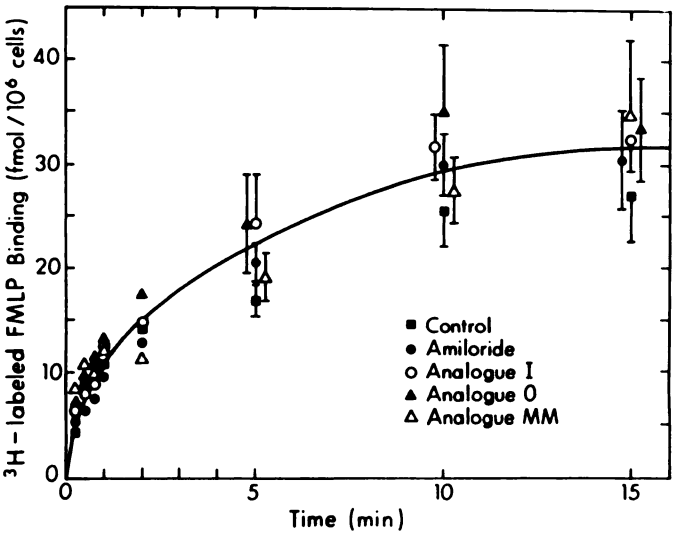


Fig. 5. Time course of ^{3}H-labeled FMLP binding to neutrophils. Cells were incubated at 37° with 10 nM ^{3}H-labeled FMLP in the presence or absence of amiloride or analogues I, O, or MM at concentrations of 1000, 1000, 100, and 10 μM, respectively. Nonspecific binding was measured in the presence of a 1000-fold excess of unlabeled FMLP. At stated times, aliquots of the neutrophil suspensions were removed and assayed for radioactivity. The resutls of three experiments are expressed in terms of specific binding, defined as total minus nonspecific binding. The *curve* through the data points was drawn by eye. To maintain clarity, the *error bars* have been omitted from the first five time points (15 sec to 2 min).

First, it appears that amiloride analogues with an unsubstituted guanidino moiety are required since any substitution of a terminal nitrogen atom results in a dramatic reduction in activity. As suggested by Vigne *et al.* (18), this may relate to the interaction of the protonated guanidino group with the Na^+ translocation site of the Na^+/H^+ exchange protein. Amiloride, by virtue of its guanidino moiety, exhibits a pK_a of ~8.7, behaves as a weak base, and, at physiological pH (7.40), exists mainly in the protonated form. There is considerable speculation that this may be the active group that recognizes and binds to the Na^+ transport site of the exchanger. This hypothesis is supported by the observations of L'Allemain *et al.* (19) using an amiloride analogue with a lower pK_a (7.03). They found that the protonated form of amiloride is required for inhibition of Na^+/H^+ exchange. These results are similar to those of Benos *et al.* (34) in their original studies of Na^+ conductance in frog skin, where they first proposed that the charged form of amiloride is essential for activity. This finding was subsequently confirmed by Cuthbert and Fanelli (32), who found that amiloride has to be protonated to be biologically active. However, we should point out that, although amiloride needs to carry a positive charge to inhibit either the Na^+/H^+ exchanger or the Na^+ channel, this finding does not necessarily imply that this part of the molecule is the actual binding site to the transporter.

Amiloride was first discovered to be an inhibitor of Na^+ channels in tight epithelia such as frog skin (32, 34, 35), where the activity of a number of the analogues used in the present study was tested. Several striking differences in the structure-activity relations of the two systems (i.e., Na^+ channels in frog skin and Na^+/H^+ exchange in neutrophils) are readily apparent. Thus, in the frog skin assay, benzamil is 9-fold more active than amiloride, phenamil 17-fold, the 2-phenethyl analogue (GGG) 14-fold, and the tertiary-octyl analogue (FFF) 15-fold more active (32, 35, 36). As seen in Table 1, these compounds are virtually inactive against the Na^+/H^+ exchanger in neutrophils.

The second structural requirement for inhibitory activity toward the Na^+/H^+ exchanger involves the 6-position substituent of amiloride. Replacement of the 6-Cl group by H or F decreases activity, whereas replacement by Br or I enhances activity. Thus, the order of activity is (I > Br > Cl > F > H), with the 6-I compound being 5-fold more active than amiloride. In contrast, the 6-Br compound is equipotent to amiloride in the frog skin assay, whereas the 6-I compound is 7-fold *less* active (32, 34).

The third and most important structural requirement for Na^+/H^+ exchange-inhibitory activity was the presence of one or two substituents on the 5-amino nitrogen atom of the amiloride molecule. With *single* substituents, increasing the number of carbon atoms (at least up to 6) produces a corresponding increase in activity. This applies to alkyl, alkenyl, alkynyl, cycloalkyl, and cycloalkylalkyl groups. Branching of alkyl groups had a beneficial effect on activity. A phenyl group moderately increased activity whereas a benzyl, substituted benzyl, or 2-phenethyl group was somewhat less effective.

Two alkyl or an alkyl and an alkenyl group had a profound effect in enhancing activity with the peak activity occurring when the total number of carbon atoms was 5–7. The best effects were observed when the two alkyl chains were joined to form a 5- to 7-atom ring. The most active compound of the

series was the 5-*N,N*-hexamethylene analogue (MM) which was ~500-fold more potent than amiloride.

The structure-activity relationships for inhibition of Na^+/H^+ exchange in human neutrophils are strikingly similar to those recently reported in chick muscle cells (18), guinea pig fibroblasts (19), and human epidermoid carcinoma cells (20). These findings confirm the strong similarities among Na^+/H^+ exchangers throughout the animal kingdom and suggest that essential elements of the primary and secondary structures of the Na^+-binding sites are conserved.

The structure-activity relationships involving the Na^+/Ca^{2+} exchanger, as seen in brain and heart muscle cells (16, 37, 38), are different from those observed for both the Na^+ channel and the Na^+/H^+ exchanger. In pituitary plasma membrane vesicles, substituents on a terminal guanidino nitrogen atom exhibited enhanced activity over amiloride. Thus, phenamil was 6-fold more active than amiloride, benzamil 11-fold, and the tertiary-octyl analogue (FFF) 8-fold more active (16), whereas these were all much less active than amiloride in inhibiting the Na^+/H^+ exchanger of neutrophils.

Substitution of the 5-amino nitrogen atom of amiloride often has some activity-enhancing effect against the Na^+/Ca^{2+} exchanger, but the structure-activity relationships are not parallel to those seen with the Na^+/H^+ exchanger. Thus, the monoallyl (KK) and propargyl (SS) compounds are only equipotent to amiloride, whereas the tertiary-butyl (DD) and cyclopentyl compounds (JJ) are, respectively, 12- and 16-fold more active than amiloride toward the Na^+/Ca^{2+} exchanger, but 26-fold and 22-fold more active than amiloride toward the Na^+/H^+ exchanger. Conversely, the 4-methylbenzyl compound (BBB) is 20-fold more active than amiloride against the Na^+/Ca^{2+} exchanger but only 10-fold more active toward the Na^+/H^+ exchanger. Among the disubstituted analogues, the 5-(*N*-propyl-*N*-butyl) compound (P) was 25-fold more active than amiloride toward the Na^+/Ca^{2+} exchanger but 226-fold against the Na^+/H^+ exchanger. Likewise, the 5-*N,N*-hexamethylene compound (MM) was only about 25-fold more active than amiloride toward the Na^+/Ca^{2+} exchanger but ~500-fold more active against the Na^+/H^+ exchanger of human neutrophils.

In summary, we have identified a number of amiloride analogues with activities 10–500-fold greater than that of the parent compound for inhibition of FMLP-activated Na^+/H^+ exchange in human neutrophils. These studies should provide useful pharmacologic tools for gaining insight into the pH_i-dependent functions of these cells.

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