Vol. 116, No. 1, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 14, 1983 Pages 86-90

# ETHYLISOPROPYL-AMILORIDE : A NEW AND HIGHLY POTENT DERIVATIVE OF AMILORIDE FOR THE INHIBITION OF THE $Na^+/H^+$ EXCHANGE SYSTEM IN VARIOUS CELL TYPES

Paul Vigne[1], Christian Frelin[1], Edward J. Cragoe Jr[2] and Michel Lazdunski[1]

[1] Centre de Biochimie du CNRS, Faculté des Sciences, Parc Valrose, 06034 Nice Cedex, France
[2] Merck, Sharp & Dohme Research Laboratories West Point, Pennsylvania 19486, USA

Received August 30, 1983

Ethylisopropyl-amiloride is 100 times more potent than amiloride for inhibiting the $Na^+/H^+$ exchanger of 3T3 fibroblasts, chick skeletal muscle cells and chick cardiac cells. Half-maximum effects, measured at 3 mM external $Na^+$ are observed at 20-100 nM and 5 µM for ethylisopropyl-amiloride and amiloride respectively. As previously observed for amiloride, the effect of ethylisopropyl-amiloride is antagonized by external $Na^+$ ions.

The $Na^+/H^+$ exchange system is a major mechanism for the regulation of internal pH in eukaryotic cells (1-2). It is inhibited by amiloride (3,5-diamino-6-chloro-N-(diaminomethylene)pyrazine-carboxamide), a well known diuretic drug (3, 4). Inhibition of the $Na^+/H^+$ exchange system by amiloride is competitively antagonized by $Na^+$ ions, so that under physiological conditions (140 mM external $Na^+$) millimolar concentrations of amiloride are required to block the $Na^+/H^+$ exchanger (3). This has prevented both a biochemical characterization of the $Na^+/H^+$ exchanger and detailed investigations on its exact role in various cellular processes such as the control of cell proliferation (5). High affinity inhibitors of ion transport systems such as tetrodotoxin, apamin, nitrendipine or ouabain have been found to be essential to analyse the molecular properties of voltage-dependent $Na^+$ channels (6), $Ca^{2+}$-dependent $K^+$ channels (7), $Ca^{2+}$ channels (8), or $(Na^+,K^+)$ATPase (9) respectively.

## Materials and Methods

Amiloride and Ethylisopropyl-amiloride (3-amino-5-ethylisopropylamino-6-chloro-N-(diaminomethylene)pyrazine-carboxamide) were synthesized as previously described (10, 11). The structures of there two compounds are presented in

0006-291X/83 $1.50

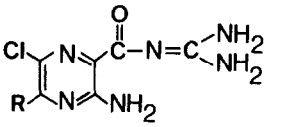

Fig. 1 Structure of amiloride (R = $NH_2$) and ethylisopropyl-amiloride (R = N - $CH_2$-$CH_3$ with $CH(CH_3)_2$)

Figure 1. Cultures of chick skeletal muscle cells, chick cardiac cells and resting cultures of 3T3 fibroblasts were prepared as previously reported (4, 11, 12) and grown in 24 wells tissue culture clusters.

An acidification of the internal cellular medium was found necessary to obtain high rates of amiloride sensitive $^{22}Na^+$ uptake (4). Transient acidification can be obtained by pretreating cells with $NH_4^+$ containing solutions (4). More stable and long lasting intracellular acidifications were obtained by using nigericin (4), an ionophore that realizes the electroneutral exchange of $K^+$ for $H^+$ (13). Therefore cells were first incubated with amiloride or its analog for 15 minutes in a $Na^+$ free, 5.4 mM $K^+$ Earle solution supplemented with 1 µg/ml of nigericin. Then measurements of $^{22}Na^+$ uptake rates after addition of $^{22}Na^+$ were performed in the absence of nigericin and under conditions which we have previously described (4). The external $Na^+$ concentrations used in this work were 3 mM or 140 mM.

## Results and Discussion

Figure 2 shows the time course of $^{22}Na^+$ accumulation by resting cultures of 3T3 fibroblasts in the presence and absence of 0.2 mM amiloride and after equilibration of the cells with 1 µg/ml of nigericin.

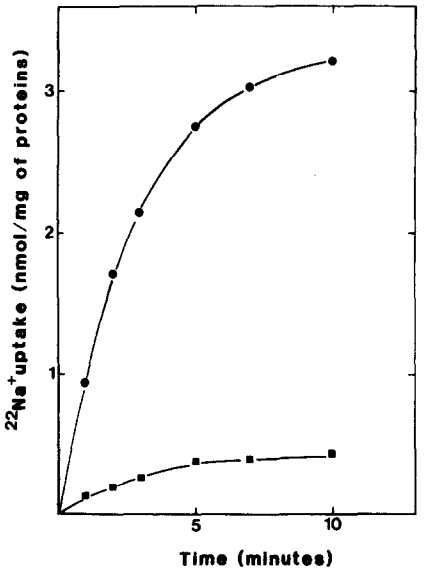


Fig. 2 The effect of amiloride on the time course of $^{22}Na^+$ accumulation by resting cultures of 3T3 cells. 3T3 cells were equilibrated for 15 minutes in a $Na^+$ free, 5.4 mM $K^+$ medium in the presence of 1 µg/ml of nigericin. $^{22}Na^+$ accumulation was then followed using a nigericin free, 3 mM $Na^+$ medium supplemented with 0.2 mM ouabain and in the presence (■) or in the absence (●) of 0.2 mM amiloride.

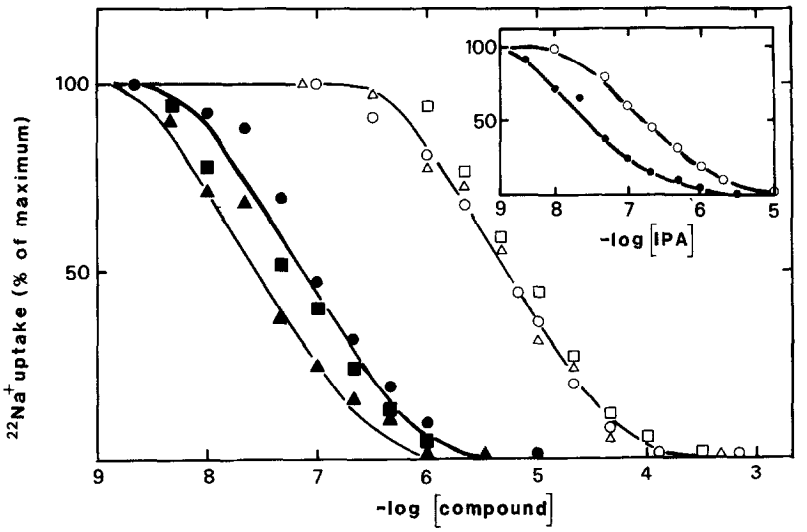


Fig. 3 Dose-response curves for amiloride and ethylisopropyl-amiloride action on the $Na^+/H^+$ exchange system. Main panel : Dose-response curves for amiloride (open symbols) and ethylisopropyl-amiloride (closed symbols) inhibition of the initial rate of $^{22}Na^+$ uptake by chick skeletal muscle cells (□,■), chick cardiac cells (△,▲) and 3T3 cells (○,●). Experiments were performed at 3 nM external $Na^+$. Inset : Dose-response curves for ethylisopropyl-amiloride (IPA) inhibition of the initial rate of $^{22}Na^+$ uptake by chick cardiac cells at 3 mM external $Na^+$ (●) and 140 mM external $Na^+$ (○).

Figure 3 shows the dose-response curve for amiloride inhibition of the initial rate of $^{22}Na^+$ uptake by 3T3 fibroblasts, chick skeletal muscle cells and chick cardiac cells.

Half-maximum inhibitions were observed near 5 μM (3T3 cells : 5 μM, chick skeletal muscle cells 7 μM, chick cardiac cells : 5 μM). The influence of a number of amiloride analogs on the rate of $^{22}Na^+$ uptake was investigated and dose-response curves were established for each of them. Substitution of the guanidine group of amiloride by various substituents produced molecules which were almost inactive for the inhibition of the $Na^+/H^+$ exchange system. More potent molecules than amiloride were obtained by substituting the 5-amino group. The most active molecule was the ethylisopropyl substituted analog shown in Figure 1 with a half-maximum effect on the rate of $^{22}Na^+$ uptake comprised between 20 nM and 100 nM depending on the cell type (3T3 cells : 100 nM, chick skeletal muscle cells : 60 nM, chick cardiac cells : 20 nM). As previously reported for amiloride (3), the inhibitory effect of ethylisopropyl-amiloride on $^{22}Na^+$ uptake is competitively antagonized by external $Na^+$.

For instance in chick cardiac cells, increasing the external $Na^+$ concentration from 3 to 140 mM shifted the half-maximum effect for ethylisopropyl-amiloride inhibition from 20 nM to 150 nM (Figure 3 inset). Similarly in chick skeletal muscle cells increasing the external $Na^+$ concentration from 3 mM to 50 mM shifted the half maximum effect for the amiloride analog from 50 nM to 150 nM.

The competition between $Na^+$ and ethylisopropyl-amiloride is expressed by the following equation

$$K_{0.5} = K_D \left( 1 + \frac{[Na^+]}{K_{Na^+}} \right)$$

where $K_D$ and $K_{0.5}$ are the true and apparent dissociation constants of the ethylisopropyl-amiloride-receptor complex, $[Na^+]$ is the external $Na^+$ concentration and $K_{Na^+}$ is the inhibitory constant for $Na^+$ ions. From this equation and with the previous values one can calculate the dissociation constant of ethylisopropyl-amiloride for its receptor site on the $Na^+/H^+$ exchanger. This value is 18 nM for chick cardiac cells and 50 nM for chick skeletal muscle cells. $K_{Na^+}$ values are 18 mM and 25 mM respectively. A value of 13 mM has been found for the amiloride binding site in chick skeletal muscle cells (3).

In application of equation 1 concentrations of 2 µM and 4 µM ethylisopropyl-amiloride block 90 % of the activity of the $Na^+/H^+$ exchanger in cardiac and skeletal muscle cells respectively under physiological conditions in which the external concentration of $Na^+$ is 140 mM.

Millimolar concentrations of amiloride would be required to produced the same effect.

In conclusion ethylisopropyl-amiloride will probably serve now as a better and more specific inhibitor than amiloride for studying the role of $Na^+/H^+$ exchanger under physiological conditions. Furthermore it is probable that radiolabeling of this amiloride analog will permit to titrate and may be to purify the molecular system which ensures $Na^+/H^+$ exchange in biological membranes.

Acknowledgements

We are grateful to M.T. Ravier, N. Boyer and M. Valetti for expert technical assistance. This work was supported by grants from the Centre National de la Recherche Scientifique, the Institut National de la Santé et de la Recherche Médicale and the Fondation pour la Recherche Médicale.

References

1. Roos, A. and Boron, W.F. (1981) Physiol. Rev. 61, 296-434.
2. Boron, A. (1983) J. Memb. Biol. 72, 1-16.
3. Vigne, P., Frelin, C. and Lazdunski, M. (1982) J. Biol. Chem. 257, 9394-9400.
4. Frelin, C., Vigne, P. and Lazdunski, M. (1983) J. Biol. Chem. 258, 6272-6276.
5. Pouysségur, J., Chambard, J.C., Franchi, A., Paris, S. and Van Obberghen-Schilling, E. (1982) Proc. Natl. Acad. Sci. 79, 3935-3939.
6. Lombet, A., Norman, R.I. and Lazdunski, M. (1983) Biochem. Biophys. Res. Comm. 114, 126-130.
7. Hugues, M., Romey, G., Duval, D., Vincent, J.M. and Lazdunski, M. (1982) Proc. Natl. Acad. Sci 79, 1308-1312.
8. Fosset, M., Jaimovich, E., Delpont, E. and Lazdunski, M. (1983) J. Biol. Chem. 258, 6086-6092.
9. Rossi, B., Ponzio, G. and Lazdunski, M. (1982) Embo J. 1, 859-861.
10. Cragoe, E.J., Woltersdorf, O.W., Bicking, J.B., Kwong, S.F. and Jones, J. H. (1967) J. Med. Chem. 10, 66-75.
11. Frelin, C., Lombet, A., Vigne, P., Romey, G. and Lazdunski, M. (1981) J. Biol. Chem. 256, 12355-12361.
12. Fosset, M., De Barry, J., Lenoir, M.C. and Lazdunski, M. (1977) J. Biol. Chem. 252, 6112-6117.
13. Pressman, B.C. (1976) Ann. Rev. Biochem. 45, 501-530.