0026-895X/93/051041-05$03.00/0

MOLECULAR PHARMACOLOGY, 44:1041-1045

Pharmacological Characterization of Stably Transfected Na^+/H^+ Antiporter Isoforms Using Amiloride Analogs and a New Inhibitor Exhibiting Anti-ischemic Properties

L. COUNILLON, W. SCHOLZ, H. J. LANG, and J. POUYSSÉGUR

Centre de Biochimie, Université de Nice-Sophia Antipolis, Parc Valrose, 06108 Nice Cedex (L.C., J.P.) and Hoechst AG, Postfach 800320, 6230 Frankfurt/M. 80, Germany (W.S., H.J.L.)

Received May 6, 1993; Accepted August 2, 1993

SUMMARY

A fibroblast mutant cell line devoid of Na^+/H^+ exchange was used to stably express cDNAs encoding the NHE1, NHE2, and NHE3 Na^+/H^+ antiporters. Pharmacological studies using amiloride and two of its 5-N-substituted derivatives, 5-N-dimethyl amiloride and 5-N-(methyl-propyl)amiloride (MPA), demonstrate that the NHE1 isoform is the ubiquitously expressed amiloride-sensitive Na^+/H^+ antiporter (K_i of 0.08 μM for MPA), whereas the NHE2 and NHE3 isoforms exhibit a lower affinity for these inhibitors (K_i of 0.5 μM and 10 μM, respectively, for MPA) and are therefore likely to be members of the epithelial Na^+/H^+ exchanger's family.

In addition, we have used this system to test a new Na^+/H^+ exchanger inhibitor possessing anti-ischemic properties on myocardial cells [(3-methylsulphonyl-4-piperidinobenzoyl) guanidine methanesulphonate]. This compound inhibits competitively NHE1 (K_i of 0.16 μM) with a much greater affinity than NHE2 and NHE3 (K_i of 5 μM and 650 μM, respectively) and therefore appears to be much more discriminative between these two classes of antiporter isoforms than the amiloride-related molecules. These results suggest an explanation for the observed difference of physiological effects between amiloride and HOE694, and identify this new inhibitor as a useful tool for studies of Na^+/H^+ exchange.

The Na^+/H^+ exchanger NHE1 is a ubiquitously expressed transmembrane protein that catalyzes the nonelectrogenic exchange of intracellular protons for external sodium with a stoichiometry of one to one and acts, therefore, as one of the main systems of intracellular pH regulation (for review, see Ref. 1). As a mediator of Na^+ and H^+ transport, it also plays a major role in the maintenance of the whole organism homeostasis since it has been reported to be involved in both in transepithelial sodium transport, bicarbonate reabsorbtion, and regulation of intracellular volume (for review, see Ref. 2). Because of these above-mentioned physiological roles, the Na^+/H^+ antiporter has been hypothesized to exert an important contribution in physiopathological processes such as essential hypertension (3) and postischemic cell death (4).

As with many other Na^+ transports, Na^+/H^+ exchange is blocked by the guanidinium monovalent cation, with an apparent K_i of about 30 mM, which is very close to the affinity of the antiporter for Na^+ (5). Since hydrated Na^+ and guanidium ions occupy the same volume, guanidinium ions that are not transported by the antiporter are hypothesized to block exchange by competition at the external Na^+ transport site. Among the numerous substituted guanidinium molecules that have been tested for their ability to block Na^+/H^+ exchange, the diuretic compound amiloride (6) and its 5-amino-substituted derivatives are at present the most useful and potent inhibitors of the antiporter (7). Despite the relatively low affinity of amiloride and its analogs for the exchanger (they inhibit the antiporter in the micromolar range, and can bind to many other proteins [8]), these competitive inhibitors have been useful pharmacological tools in that they have allowed the characterization of distinct Na^+/H^+ antiporter isoforms.

In addition to the ubiquitously expressed amiloride-sensitive and growth factor-activatable Na^+/H^+ exchanger, apically expressed isoforms of antiporter exhibiting a decreased affinity for amiloride and its 5-amino-substituted derivatives have been pharmacologically characterized in epithelia (cultured porcine [9] and opossum kidney cells [10], rabbit ileal villus [11], and rat colon [12]), whereas a totally amiloride-insensitive exchanger has been described in cultured hippocampal neurones (13). These antiporters have been hypothesized to be involved in specialized functions, such as sodium transepithelial transport

This work was supported by grants from the Centre National de la Recherche Scientifique (CNRS UMR 134), the Institut National de la Santé et de la Recherche Médicale (INSERM), the Fondation pour la Recherche Médicale, the Association pour la Recherche contre le Cancer (ARC), and the Association Franco-Israelienne pour la Recherche Scientifique et Technique (AFIRST).

ABBREVIATIONS: DMA, 5-N-dimethyl amiloride; MPA, 5-N-(methyl-propyl)amiloride; HOE694, (3-methylsulphonyl-4-piperidinobenzoyl) guanidine methanesulphonate.

in intestine and kidney, urine acidification coupled to bicarbonate reabsorbtion (for review see Ref. 14), as well as the maintenance of a dynamic proton equilibrium across the plasma membrane of hippocampal neurones (13).

Although the use of defined inhibitors has been very useful for the characterization of different Na^+/H^+ exchanger isoforms, several important questions remain to be answered. First, IC_{50} of amiloride for Na^+/H^+ exchange had given differing results in respect to the cell systems in which it had been investigated (e.g., 27 μM in apical membranes of rat colon [12] and 425 μM in rat colonic brush-border vesicles [15]). Therefore, it was possible to hypothesize that there were distinct amiloride-resistant isoforms expressed in different epithelial cell types, but this remained to be proven. Second, no structural information concerning these putative isoforms was available.

The cloning of cDNA coding for the amiloride-sensitive Na^+/H^+ antiporter (16) provided the means to answer these questions. Its use as a probe to screen cDNA libraries prepared from epithelial tissues of kidney and gastrointestinal tract allowed the cloning of three distinct Na^+/H^+ isoforms termed NHE2 (16a, 16b), NHE3 (17, 18), and NHE4 (17). We have used a fibroblast cell line lacking Na^+/H^+ exchange activity (19) to express the cDNAs coding for the NHE1, NHE2, and NHE3 isoforms and determine their pharmacological profiles. In this article, we present the evidence that these cDNAs encode functional Na^+/H^+ antiporters exhibiting very distinct pharmacological profiles that are reminiscent of those that had been determined in epithelial cells. In addition, we have tested a new anti-ischemic guanidinium derivative that appears to be more discriminative for the different isoforms than the amiloride-related compounds.

Material and Methods

Cell culture. Chinese hamster fibroblast cells (CCL39 cell line), the CCL39-derived PS120 variant (19), and PS120 cells transfected with cDNAs coding for the different Na^+/H^+ exchanger isoforms were maintained in Dulbecco's modified Eagle's medium supplemented with 7.5% fetal calf serum, penicillin (50 units/ml), and streptomycin (50 μg/ml) in a humidified athmosphere of 5% CO_2 and 95% air at 37°C.

Expression of Na^+/H^+ antiporters. The coding regions of the NHE1 and NHE2 cDNAs were ligated into the cloning site of the pECE mammalian expression vector (20).

PS120 cells were transfected using the calcium phosphate precipitation method (21). Positive transfectants were selected by their ability to survive a 1-hr long intracellular acidification induced by $NH4^+$ acid loading. As described elsewhere (16), this procedure kills 100% of the control untransfected population but allows survival of cells expressing Na^+/H^+ exchange activity. The procedure was repeated twice a week until stable antiporter activity was observed. Pharmacological experiments were performed on cell populations obtained by pooling the acid-resistant colonies. This method of selection allowed the isolation of cell populations expressing NHE1, NHE2, and NHE3 isoforms with similar levels of Na^+/H^+ exchange activity (the observed differences did not exceed 3-fold).

$^{22}Na^+$ uptake studies. Amiloride, DMA, and MPA were solubilized in dimethyl sulfoxide and diluted in the uptake medium at the appropriate concentrations just before use. Care was taken to avoid dimethyl sulfoxide cellular toxicity by diluting stock solutions at least 100 times in the aqueous uptake medium. HOE694 was dissolved in sterile distilled water and diluted as mentioned above. Stability of the inhibitors was checked periodically by measurement of the previously determined K_i for the NHE1 isoform expressed by the Chinese hamster fibroblast CCL39 cell line.

Cells were grown to confluency in 24-well plates and incubated for 60 min at 37°C in a medium containing 50 mM NH_4Cl and buffered at pH 7.0 with 15 mM 4-morpholinepropanesulfonic acid. Cells were then washed twice in less than 15 sec with 120 mM choline chloride medium buffered at pH 7.4 with 15 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-Tris and loaded with the uptake medium containing 0.2 μCi of carrier-free $^{22}Na^+$/ml (Amersham), 1 mM ouabain, 1 mM $MgCl_2$, 2 mM $CaCl_2$, and 120 mM choline chloride, and buffered at pH 7.4 with 15 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-Tris. In these conditions, $^{22}Na^+$ uptake was shown to be linear for more than 6 min; therefore, all the dose-response curves for amiloride and derivatives were measured in uptake experiments of 6 min duration. In experiments involving competition with nonradioactive external sodium, uptake time was reduced to 30 sec to ensure initial rate conditions (Fig. 2).

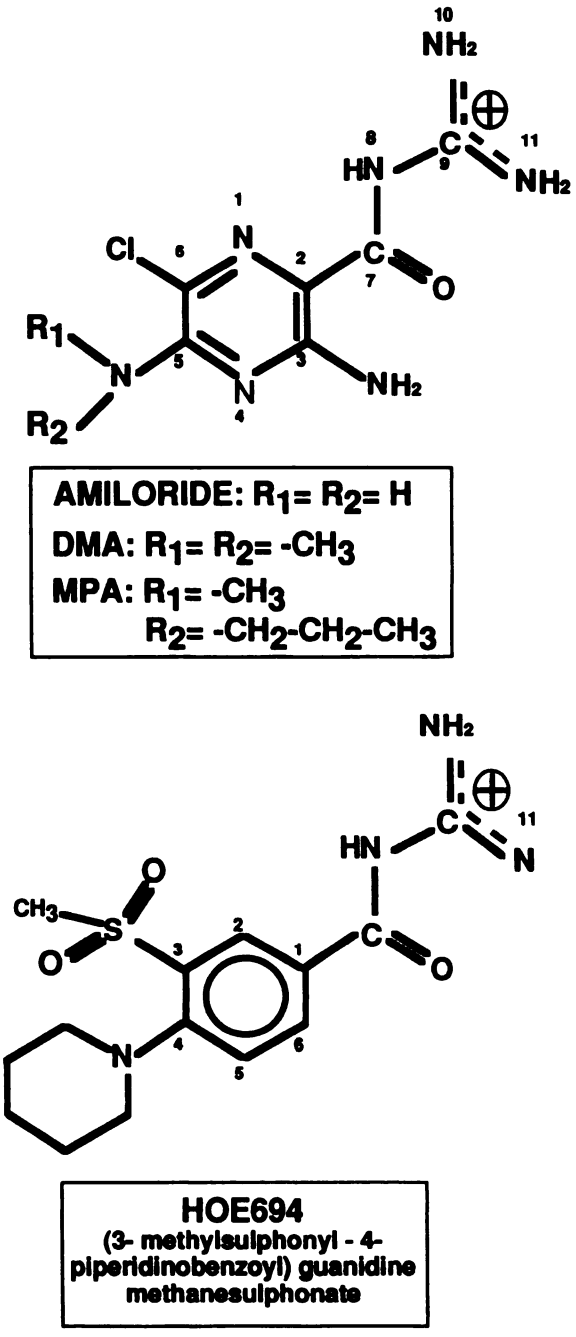

Fig. 1. The chemical structure of amiloride, 5-*N*-diméthyl amiloride, 5-*N*-(methyl-propyl) amiloride, and HOE694 (3-methylsulfonyl-4-piperidinobenzoyl) guanidine methanesulfonate.

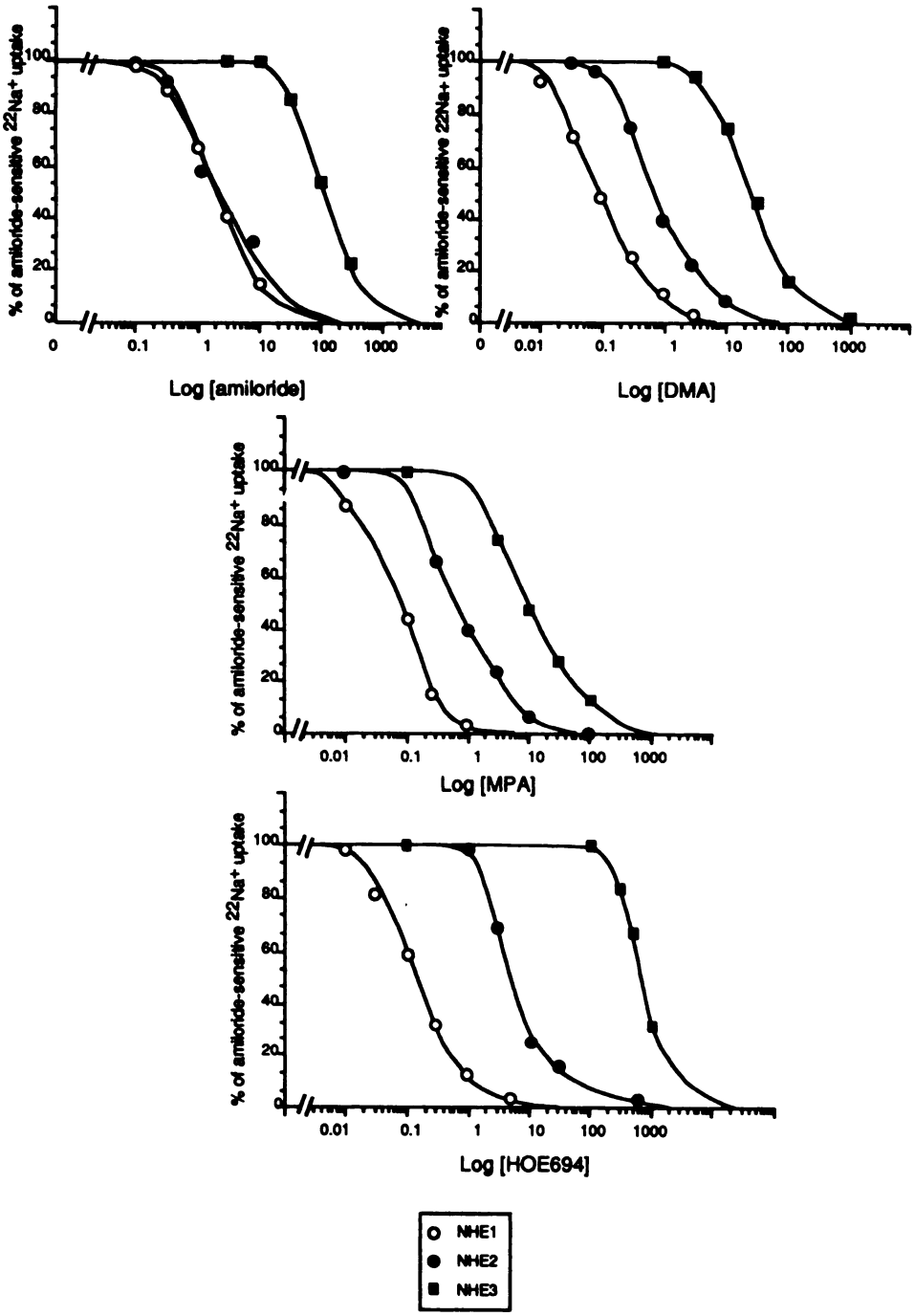

Fig. 2. Dose-response curves for inhibition of initial rates of amiloride-sensitive $^{22}Na^+$ uptake. As described in Materials and Methods, increased concentrations of amiloride, DMA, MPA, and HOE694 were assayed to inhibit initial rates of $^{22}Na^+$ influx mediated by the NHE1, NHE2, and NHE3 isoforms expressed in antiporter-deficient PS120 fibroblasts.

TABLE 1

***K_i* values for amiloride, DMA, MPA, and HOE694 as determined by dose-dependent inhibition of amiloride-sensitive $^{22}Na^+$ uptake mediated by NHE1, NHE2, and NHE3 isoforms**

	Amiloride	DMA	MPA	HOE694
	(μM)	(μM)	(μM)	(μM)
NHE1	3	0.1	0.08	0.16
NHE2	3	0.7	0.5	5
NHE3	100	11	10	650

Influx of $^{22}Na^+$ was stopped by rinsing the cell monolayer four times with phosphate-buffered saline at 0°. Cells were solubilized in 0.1 N NaOH and radioactivity was assayed by liquid scintillation spectrometry. Amiloride-sensitive $^{22}Na^+$ influx was determined as the difference between the initial rates of 22 Na^+ uptake measured in the absence and in the presence of 500 μM of MPA.

Results

Inhibition of the NHE1 isoform by HOE694. As shown in Fig. 1, HOE694 is a substituted benzoyl guanidine that exhibits a similar structure to amiloride. The guanidinium group acts as the sodium competitor, while the substituted aromatic ring enhances its affinity for the Na^+/H^+ exchanger. Therefore, it was tempting to hypothesize that this compound would have a similar mechanism of action as 5-amino-substituted derivatives of amiloride. $^{22}Na^+$ uptake experiments indicate that the apparent affinity of the NHE1 isoform for Na^+ is

decreased by HOE694, whereas V_{max} is not significantly modified by this inhibitor (data not shown). These results indicate that this compound likely behaves as a competitor for sodium at the NHE1 external transport site, as shown previously for amiloride and its 5-amino-substituted derivatives on the NHE1 isoform.

Compared effects of HOE694 and amiloride series inhibitors on NHE1, NHE2, and NHE3. We assayed increasing concentrations of amiloride, DMA, MPA, and HOE694 for their ability to inhibit initial rates of amiloride-sensitive $^{22}Na^+$ uptake in antiporter-deficient PS120 cells transfected with the exchanger isoforms. The obtained dose-response curves (Fig. 2) provide the following information.

First, amiloride, DMA, and MPA inhibit the transfected NHE1 isoform with the same K_i value as those obtained previously (7, 22). This result confirms that this cDNA encodes the ubiquitously expressed amiloride-sensitive Na^+/H^+ exchanger. The transfected NHE2 exhibits a similar affinity for amiloride, but a about 10-fold lower affinity for DMA and MPA when compared with NHE1. These results suggest that a similar amiloride binding site topology is shared by NHE1 and NHE2, whereas the region of these proteins binding the 5-*N* substituents of this inhibitor are different. To a greater extent, the NHE3 isoform displays a lower sensitivity for amiloride, as well as for its *N*-substituted derivatives (about a 100-fold difference for MPA between NHE1 and NHE3). We can deduce, therefore, that these recently cloned cDNAs may encode members of the amiloride-resistant Na^+/H^+ exchanger family which had been discovered initially in epithelial cells.

Second, for each expressed antiporter, the 5-amino-substituted amiloride derivatives MPA and DMA appear to be more potent inhibitors than amiloride itself. The finding that the order of potency of these compounds remains the same for every isoform indicates that their mechanism of interaction with the different antiporters remains unchanged, despite the observed changes of affinity. More interestingly, HOE694, which behaves as a true amiloride 5-*N* derivative in respect to the NHE1 isoform, is a much less potent inhibitor of both NHE2 and NHE3 than amiloride. Moreover, the affinity of this compound is very low for the putative epithelial isoforms when compared with NHE1 (more than a 4000-fold difference between NHE1 and NHE3). This result indicates that although HOE694 shares the same mechanism of competitive inhibition with amiloride, it may not be in contact with exactly the same amino acid residues as amiloride and its derivatives within the NHE2 and NHE3 isoforms.

Discussion

The above-mentioned results provide important information both on the characterization of the recently cloned Na^+/H^+ antiporter isoforms, and on the features of a new kind of Na^+/H^+ exchange inhibitor.

The molecular cloning of cDNAs coding for Na^+/H^+ exchanger isoforms and their subsequent expression in antiporter-deficient cells has allowed the pharmacological characterization of the encoded antiporters as "amiloride-resistant" isoforms (Table 1). Based on the low affinity for amiloride and its derivatives, we strongly suggest that these antiporters are the previously described epithelial Na^+/H^+ exchangers. This hypothesis is reinforced by the kidney and gastrointestinal tract distribution of mRNA encoding these two isoform mRNAs (16a, 16b, 17) as well as their apical targeting when the corresponding cDNAs are expressed in epithelial cells.[1,2]

The benzoylguanidine derivative HOE694 was originally designed to be a potent NHE1 blocker possessing anti-ischemic properties on heart cells. It had been hypothesized previously that as a major mechanism of internal pH regulation in acidified anoxic myocardial cells, Na^+/H^+ exchange (NHE1 isoform) would greatly enhance sodium ion entry, thereby triggering increased adenosine triphosphate consumption by the Na^+/K^+ATPase, a well as a massive Ca^{2+} entry via the Na^+/Ca^{2+} exchanger (4). These two events would result in both cell damage and myocardial arrhythmias during reperfusion. Hence, any potent NHE1 blocker would have beneficial effects on these direct consequences of myocardial infarction (23). HOE694 indeed exhibits all these features and can strongly reduce the arrhythmia frequency as well as cell damage on isolated rat hearts that have undergone partial coronary ligation (24). This compound is, therefore, a promising candidate for clinical application. Amiloride 5-*N*-substituted derivatives, possess similar antiarythmic properties (23), but also exhibit side effects *in vivo*, such as a negative chronotropic effect (25), which is not observed upon HOE694 administration. Further evidence indicating that these two classes of compound have a different mode of action on the whole body is the absence of diuretic effects for HOE694, whereas amiloride is clinically used as a diuretic drug (26). A possible explanation for these differences could come, at least in part, from the results reported in this paper. We have shown that HOE694 has a similar pharmacological profile of Na^+/H^+ exchanger inhibition as that demonstrated by 5-*N*-substituted derivatives of amiloride in respect to the NHE1 isoform, which is expressed in the heart, whereas epithelial NHE isoforms are much less sensitive to this inhibitor than to amiloride-related molecules. It is, therefore, very likely that *in vivo* HOE694 can specifically inhibit the heart NHE1 isoform, whereas the epithelial Na^+/H^+ exchangers remain free of inhibition.

More fundamentally, the highly discriminative potency of this compound makes it a very powerful tool for the investigation and characterization of presently unidentified Na^+/H^+ isoforms. In addition it provides a means to specifically antagonize the NHE1 isoform in most cells, permitting the use of NHE3, a HOE694-resistant isoform, as a new selective marker in gene transfer.[3]

Acknowledgments

We are grateful to Dr. Fergus McKenzie for critical reading of the manuscript. We also thank Drs. M. Donowitz and J. Orlowski for providing the NHE2 and NHE3 cDNas, respectively.

References

1. Aronson, P., and W. Boron, ed. *Na^+/H^+ Exchange, Intracellular pH and Cell Function*. Academic Press, New York (1986).
2. Grinstein, S., ed. *Na^+/H^+ Exchange*. CRC Press, Boca Raton, FL (1988).
3. Canessa, M., M. Keven, and A. Semplicini. Genetic difference in lithium-sodium exchange and regulation of the sodium hydrogen exchanger in essential hypertension. *J. Cardiovasc. Pharmacol.* **2**:592–598 (1988).
4. Lazdunski, M., C. Frelin, and P. Vigne. The sodium/hydrogen exchange system in cardiac cells. Its biochemical and pharmacological properties and its role in regulating internal concentrations of sodium and internal pH. *J. Mol. Cell. Cardiol.* **17**:1029–1042 (1985).
5. Frelin, C., P. Vigne, P. Barbry, and M. Lazdunski. Interaction of guanidinium and guanidinium derivatives with the Na^+/H^+ exchange system. *FEBS Lett.* **154**:241–245 (1986).

[1] M. Donowitz, personal communication.
[2] D. Roux, unpublished results.
[3] L. Counillon and J. Pouysségur, manuscript in preparation.

6. Benos, D. Amiloride: a molecular probe for sodium transport in tissues and cells. *Am. J. Physiol.* **242:**C131-C145 (1982).
7. L'Allemain, G., A. Franchi, E. J. Cragoe, and J. Pouysségur. Blockade of the Na^+/H^+ antiport abolishes growth factor-induced DNA synthesis in fibroblasts. *J. Biol. Chem.* **259:**4313–4319 (1984).
8. Kleyman, T. R., and E. J. Cragoe. Amiloride and its analogs as tools in the study of ion transport. *J. Membr. Biol.* **105:**1–21 (1988).
9. Haggerty, J. G., N. Agarwal, R. F. Reilly, E. A. Adelberg, and C. W. Slayman. Pharmacologically different Na/H antiporters on the apical and basolateral surfaces of cultured porcine kidney cells (LLC-PK_1). *Proc. Natl. Acad. Sci. USA* **85:**6797–6801 (1988).
10. Ramamoorthy, S., C. Tiruppathi, C. N. Nair, V. B. Mahesh, F. H. Leibach, and V. Ganapathy. Relative sensitivity to inhibition by cimetidine and clonidine differentiates between the two types of Na^+/H^+ exchangers in cultured cells. *Biochem. J.* **280:**317–322 (1991).
11. Knickelbein, R., P. S. Aronson, W. Atherton, and J. Dobbins. Sodium and chloride transport across rabbit ileal brush-border. Evidence of Na^+/H^+ exchange. *Am. J. Physiol.* **245:**G504-G510 (1983).
12. Vazhaikkurichi, M., R. Binder, and J. Binder. Characterization of Na-H exchange in apical membrane vesicles of rat colon. *J. Biol. Chem.* **265:**8408–8414 (1990).
13. Raley-Susman, K. M., E. J. Cragoe, R. M. Sapolsky, and R. R. Kopito. Regulation of intracellular pH in cultured hippocampal neurones by an amiloride-insensitive Na^+/H^+ exchanger. *J. Biol. Chem.* **266:**2739–2745 (1991).
14. Burckhardt, B.-C., and Burckhardt, G. Cellular mechanisms of proximal tubular acidification, in *pH Homeostasis* (D. Häussinger, ed.) Academic Press, New York, 233–257 (1988).
15. Foster, E. S., P. K. Dujeda, and T. A. Brasitus. Na^+/H^+ exchange in rat colonic brush-border vesicles. *Am. J. Physiol.* **250:**G781-G787 (1986)
16. Sardet, C., A. Franchi, and J. Pouysségur. Molecular cloning, primary structure, and expression of the human growth factor-activatable Na^+/H^+ antiporter. *Cell* **56:**271–280 (1989).
16a. Tse, C. M., S. A. Levine, C. H. C. Yun, M. H. Montiose, P. J. Little, J. Pouysségur, and M. Donowitz. Cloning and expression of a rabbit cDNA encoding a serum-activated ethylisopropylamiloride-resistant Na^+/H^+ exchanger isoform (NHE-2). *J. Biol. Chem.* **268:**'11917–11924 (1993).
16b. Wang, Z., J. Orlowski, and G. E. Shull. Primary structure and functional expression of a novel gastrointestinal isoform of the rat Na/H exchanger. *J. Biol. Chem.* **268:**11925–11928 (1993).
17. Orlowski, J., R. A. Kandasamy, and G. E. Shull. Molecular cloning of putative members of the Na^+/H^+ exchanger gene family. *J. Biol. Chem.* **267:**9331–9339 (1992).
18. Tse, C. M., S. R. Brant, S. Walker, J. Pouysségur, and M. Donowitz. Cloning and sequencing of a rabbit cDNA encoding an intestinal and kidney specific Na^+/H^+ exchanger isoform (NHE-3). *J. Biol. Chem.* **267:**9340–9346 (1992).
19. Pouysségur, J., C. Sardet, A. Franchi, G. L'Allemain, and S. Paris. A specific mutation abolishing Na^+/H^+ antiport activity in hamster fibroblasts precludes growth at neutral and acidic pH. *Proc. Natl. Acad. Sci. USA* **81:**4833–4837 (1984).
20. Ellis, L., E. Clauser, D. O. Morgan, M. Edery, R. A. Roth, and W. J. Rutter. Replacement of insulin receptor tyrosine residues 1162 and 1163 compromises insulin-stimulated kinase activity and uptake of 2-deoxyglucose. *Cell* **45:**721–732 (1986).
21. Wigler, M., R. Sweet, G. K. Sim, B. Wold, A. Pellicer, E. Lacy, T. Maniatis, S. Silverstein, and R. Axel. Transformation of mammalian cells with genes from prokaryotes and eukaryotes. *Cell* **16:**777–785 (1979).
22. Vigne, P., C. Frelin, E. J. J. Cragoe, and M. Lazdunski. Structure-activity relationships of amiloride and certain of its analogues in relation of the blockade of the Na^+/H^+ exchange system. *Mol. Pharmacol.* **25:**131–136 (1984).
22a. Tse, M., S. Levine, C. Yun, S. Blant, L. Counillon, J. Pouysségur, and M. Donowitz. Structure/function studies of the epithelial isoforms of the mammalian Na^+/H^+ exchanger gene family. *J. Membrane Biol.* **135:**93–108 (1993).
23. Scholz, W., U. Albus, W. Linz, P. A. Martorana, H. J. Lang, and B. A. Schölkens. Effects of Na+/H+ exchange inhibitors in cardiac ischemia. *J. Mol. Cell. Cardiol.* **24:**733–741 (1992).
24. Scholz, W., U. Albus, H. J. Lang, W. Linz, P. A. Martorana, H. C. Englert, and B. A. Schölkens. HOE 694—a new Na^+/H^+ exchange inhibitor and its effects in cardiac ischemia. *Br. J. Pharmacol.* **109:**562–568 (1993).
25. Imai, S., A. Y. Shi, T. Ishibashi, and M. Nakazawa. Na^+/H^+ is not operative under low-flow ischemic conditions. *J. Mol. Cell. Cardiol.* **23:**505–517 (1991).
26. Cragoe, E. J., ed. *Structure-activity relationships in the amiloride serie*. Urban & Schwarzenberg, Baltimore-Munich (1979).

Send reprint requests to: Dr. L. Counillon, Centre de Biochimie, Université de Nice-Sophia Antipolis, Parc Valrose, 06108 Nice Cedex, France.