Vol. 175, No. 1, 1991
February 28, 1991

BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 311-317

INHIBITION OF THE RAT RENAL Na^+/H^+ EXCHANGER BY ß-ADRENERGIC ANTAGONISTS

T. Friedrich* and G. Burckhardt**

*BASF-Ludwigshafen, Hauptlabor, D-6700 Ludwigshafen, F. R. G.

**Max-Planck-Institut für Biophysik, Kennedyallee 70,
D-6000 Frankfurt/Main 70, F. R. G.

Received January 16, 1991

The ß-adrenergic antagonists, alprenolol and propranolol, inhibit the Na^+/H^+ exchanger in rat renal brush-border membrane vesicles. Half-maximal inhibition occurs at 86 μM alprenolol and 36 μM propranolol. Similar to amiloride and Na^+, propranolol protects the Na^+/H^+ exchanger from irreversible inhibition by the carboxyl group reagent, *N,N'*-dicyclohexyl-carbodiimide (DCCD). Protection is incomplete, depends on propranolol concentration, and reaches a maximum at 0.4 mM propranolol. With a comparable dose dependence, propranol protects a 65 kDa band from labeling with [^{14}C]DCCD. The data indicate that ß-adrenergic antagonists specifically interact with the proximal tubular Na^+/H^+ exchanger.

Na^+/H^+ exchangers (antiporters) in the brush-border membrane of proximal tubule cells export hydrogen ions (H^+) from the cytosol into the tubular lumen (1). Acidification of the tubular fluid plays an important role in the reabsorption of salt and water. The Na^+/H^+ exchanger in the brush-border membrane is well characterized with respect to cation specificity, modes of electroneutral cation exchange, allosteric modification by hydrogen ions, and sensitivity to inhibitors (2, 3). The prototype inhibitor of Na^+/H^+ exchangers is the diuretic amiloride. This compound which in submicromolar concentrations blocks sodium channels in distal nephron segments, inhibits the proximal tubular Na^+/H^+ exchanger in submillimolar doses (4). Hydrophobic substituents at the nitrogen in position 5 of the pyrazine ring increase the inhibitory potency indicating a two-site interaction with the antiporter: The cationic guanidino group of amiloride can bind to a negatively charged side group of the Na^+/H^+ exchanger protein, whereas the pyrazine ring with its substituents may interact with neighbouring hydrophobic portions of the protein (3-5).

If the two-site interaction model is valid, other amphiphilic cations should also interact with the Na^+/H^+ exchanger. Indeed, the α_2-adrenergic receptor agonist, clonidine, and the H_2 histamine receptor antagonist, cimetidine, inhibit Na^+/H^+ exchanger from rabbit renal (6) and human placental brush-border membranes (7,8). Moreover, a series of imidazoline and guanidinium compounds including potent antihypertensive drugs have been described as

0006-291X/91 $1.50

inhibitors of Na^+/H^+ exchangers in chick cardiac cells (9) and in an intestinal cell line, HT29 (10). In these experiments, guanochlor proved five times more potent than amiloride in inhibiting the antiporter.

Interestingly, amiloride not only inhibits Na^+/H^+ exchanger but also decreases the binding of ligands to α- and ß-adrenergic receptors in a variety of cells and membranes (11-13), and to purified (14) and in vitro expressed α_2-receptors (15). The interaction between amiloride and ligands occurs within the hydrophobic, membrane-spanning moiety of the α_2-receptor (16) and suggests a two-site interaction of ligands and other organic cations also with adrenergic receptors. Since, therefore, adrenergic receptors and Na^+/H^+ exchangers seem to share ligand binding properties, we tested whether besides clonidine also other adrenergic ligands interact with the antiporter. Here we report that ß-adrenergic antagonists inhibit the rat renal Na^+/H^+ exchanger and protect it from irreversible modification with *N,N'*-dicyclohexyl-carbodiimide (DCCD).

METHODS

Brush-border membrane vesicles were isolated from rat kidney cortex by a Mg^{2+} precipitation technique (17). Na^+/H^+ exchanger activity was determined by suspending brush-border membrane vesicles loaded with "Na-buffer" (100 mM NaCl, 50 mM KCl, 5 mM Hepes/Tris, pH 7.0) in a "TMA-buffer" (100 mM tetramethylammonium chloride, 50 mM KCl, 5 mM Hepes/Tris, pH 7.0) containing 6 μM of the ΔpH indicator, acridine orange. Intravesicular H^+ uptake (acidification) was determined by recording changes in the acridine orange absorption (493 nm; reference wavelength 525 nm). Covalent modification of the Na^+/H^+ exchanger by *N,N'*-dicyclohexylcarbodiimide (DCCD) was performed as described earlier (5) by pre-incubating brush-border vesicles for 30 min at room temperature in TMA-buffer supplemented with 150 nmol DCCD/mg membrane protein and further agents as indicated in the figure legends. Thereafter, the vesicles were washed and loaded with Na-buffer for determination of Na^+/H^+ exchanger activity. For affinity labeling 5 nmol [^{14}C]DCCD/mg protein was used in TMA-buffer. After labeling, membrane proteins were separated by discontinuous sodium dodecylsulfate polyacrylamide gel electrophoresis using tube gels (0.5 mg protein/tube). The incorporated radioactivity was determined by liquid scintillation counting of 2 mm gel slices.

RESULTS

Rat renal brush-border membrane vesicles were loaded with Na^+, suspended in a Na^+-free medium, and the resulting intravesicular acidification due to Na^+/H^+ exchange was recorded in the absence or presence of the ß-adrenergic antagonists, alprenolol and propranolol, using acridine orange as ΔpH indicator. Figure 1 shows that alprenolol and propranolol inhibit Na^+/H^+ exchange in a dose-dependent fashion. Half-maximal inhibition occurs at 85.6 $\pm$ 18.4 μM alprenolol and at 36.3 $\pm$ 5.9 μM propranolol, respectively (means $\pm$ SD from 3 membrane preparations). At 1 mM concentration, the agonists epinephrine and isoprenaline do not inhibit the renal Na^+/H^+ exchanger (data not shown).

Next we investigated whether ß-adrenergic antagonists protect the Na^+/H^+ exchanger from irreversible inhibition by the carboxyl group reagent, *N,N'*-dicyclohexylcarbodiimide

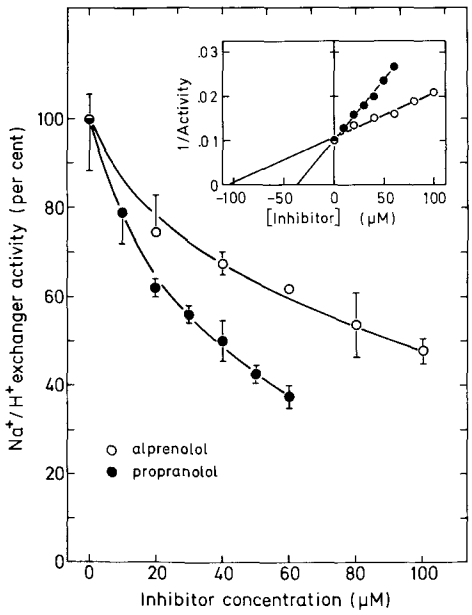

Figure 1. Inhibition of Na^+/H^+ exchange in rat renal brush-border membrane vesicles by ß-adrenergic antagonists. Vesicles were loaded with Na^+ and suspended into Na^+-free buffers containing the ΔpH indicator, acridine orange, and indicated concentrations of propranolol and alprenolol. The initial rate of Na^+/H^+ exchanger was estimated from the drop in acridine orange absorbance. Shown are means ± SD from 3-5 determinations with a single membrane preparation. The *inset* shows the same data in a Dixon plot.

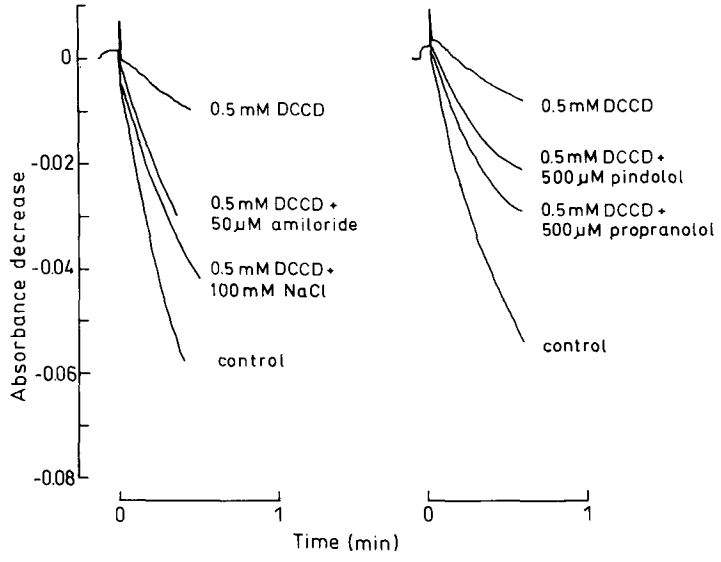

Figure 2. Protection of the Na^+/H^+ exchanger by Na^+, amiloride (left), and pindolol, propranolol (right) from irreversible inactivation by *N,N'*-dicyclohexylcarbodiimide (DCCD). Renal brush-border membrane vesicles were preincubated in TMA-buffer without (control) or with 150 nmol DCCD/mg protein and the agents indicated at the curves. Where indicated, tetramethylammonium in the preincubation buffer was replaced by Na^+. After washing, the vesicles were loaded with Na-buffer and suspended at zero time in TMA-buffer containing acridine orange. The figure shows original traces of acridine orange absorbance decrease as a function of time.

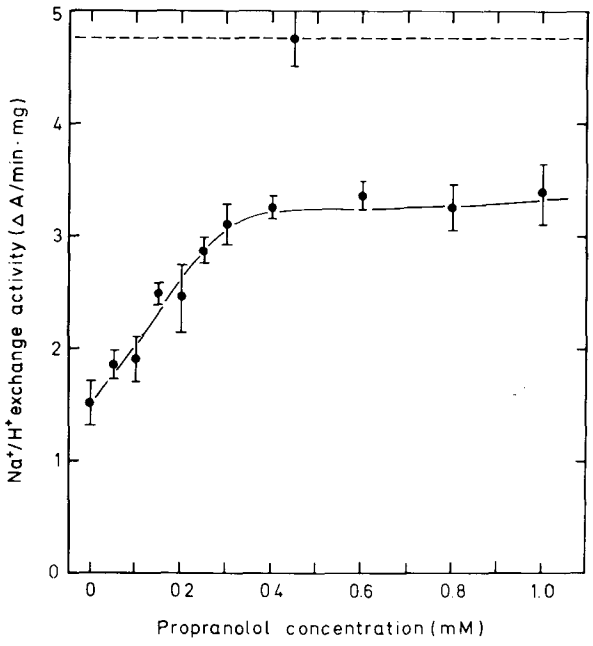

Figure 3. Dose-dependence of the protection of the Na^+/H^+ exchanger by propranolol. Vesicles were incubated for 30 min in TMA-buffer without DCCD (control; dotted horizontal line) or with 150 nmol DCCD/mg protein (closed symbols, continuous line) and the propranolol concentrations indicated on the abscissa. Thereafter the vesicles were washed, loaded with Na^+ and Na^+/H^+ exchange was determined with acridine orange. The figure shows means ± SD from 4 measurements on a single membrane preparation.

(DCCD). Figure 2, left panel, shows a marked inhibition of Na^+/H^+ exchange in DCCD-treated vesicles as compared to untreated controls indicating covalent modification of the antiporter. As found earlier (5,18,19), amiloride and Na^+ present during preincubation of vesicles with DCCD protect the antiporter partially from inactivation. Figure 2, right panel, shows that also the ß-adrenergic antagonists, pindolol and propranol, provide partial protection of the Na^+/H^+ exchanger from modification by DCCD.

The degree of protection by propranolol is dose-dependent (Figure 3). Irreversible inhibition of Na^+/H^+ exchange by 0.5 mM DCCD is the smaller (=protection is the greater) the higher the propranolol concentration is during the treatment of vesicles with the carboxyl group reagent. The protection is incomplete and reaches a maximal level at around 0.4 mM propranolol.

Protection from inactivation of the Na^+/H^+ exchanger should be reflected by a decreased affinity labeling of the related protein with [^{14}C]DCCD. Figure 4 shows that [^{14}C]DCCD is incorporated into 5 bands labeled *a* through *e*. Only the incorporation of [^{14}C]DCCD into band *d* the peak of which corresponds to an apparent mass of 65 kDa, is affected by increasing propranolol concentrations (inset). In earlier studies, the same band was protected by amiloride from labeling with [^{14}C]DCCD suggesting a relation to the renal Na^+/H^+ exchanger (5).

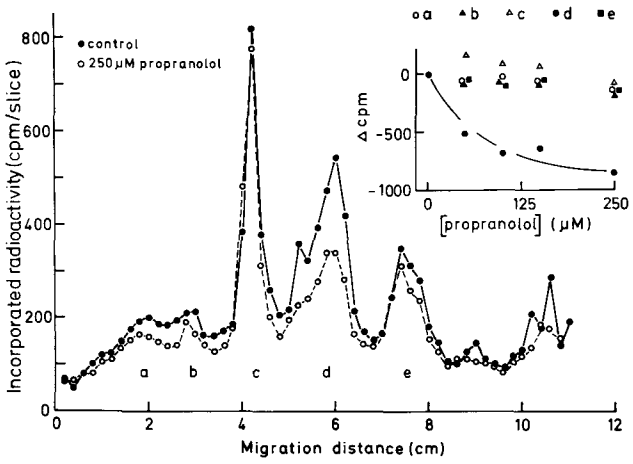

Figure 4. Effect of propranolol on affinity labeling of rat renal brush-border membrane vesicles with [^{14}C]DCCD. Vesicles were incubated with [^{14}C]DCCD in the absence or presence of the indicated propranolol concentrations. Membrane proteins were separated by SDS polyacrylamide gel electrophoresis (rod gels; 8.5% acrylamide). The incorporated radioactivity is shown as a function of the migration distance. Labels *a* through *e* refer to the apparent M_r (in kDa) of 130; 114; 89; 65; and 51, respectively. The *inset* shows the effect of increasing propranolol concentrations on the radioactivity contained in each band. Negative Δcpm denote a decrease in radioactivity compared to control membranes (no propranolol). The relation of the symbols to bands *a* through *e* is shown at the top of the inset. Shown are means from 3 membrane labeling experiments.

DISCUSSION

The ß-adrenergic antogonists, alprenolol and propranolol, inhibit the Na^+/H^+ exchanger in rat renal brush-border membrane vesicles as demonstrated in experiments with the ΔpH indicator, acridine orange. Half-maximal inhibition occurs at 86 and 36 µM, respectively. Of importance, the inhibitory potency is comparable to that of amiloride (43 µM; ref. 5) determined with the same method.

The observed inhibition by ß-adrenergic antagonists may be apparent or non-specific. We consider this possibility, however, unlikely for the following reasons. *First*, the antagonists do not dissipate a preformed ΔpH indicating that their inhibition of Na^+/H^+ exchange is not due to an action as protonophores (data not shown). *Second*, ß-antagonists do not interfere in the applied doses with acridine orange excluding an effect on the indicator system as a reason for the decrease in Na^+/H^+ exchange signals (data also not shown). *Third*, the antagonists attenuate the irreversible inhibition of the antiporter by the carboxyl group reagent, *N,N'*-dicyclohexylcarbodiimide (DCCD). Since in this type of experiment Na^+/H^+ exchange is measured after unreacted DCCD and antagonists have been washed out, non-specific effects of antagonists on vesicle integrity, H^+-permeability and on the acridine orange test system can be excluded with certainty. We rather have to assume a direct interaction of antagonists with the Na^+/H^+ exchanger.

It is not clear at which site the ß-adrenergic antagonists, alprenolol and propranolol, interact with the Na^+/H^+ exchanger. Since both compounds are amphiphilic cations similar to

amiloride, an interaction with the Na^+-binding site seems probable. Demonstration of a competitive inhibition would strongly support such a notion. In our experiments, however, we offer Na^+ from the inside and the ß-adrenergic antagonists from the outside of the vesicles and measure Na^+ efflux-driven H^+ uptake. In such a setting, a putative competition between Na^+ and antagonists can not be tested.

Propranolol and pindolol are able to decrease the irreversible inhibition of the Na^+/H^+ exchanger by DCCD. This carboxyl group reagent modifies the renal Na^+/H^+ exchanger in a Na^+- and amiloride-protectable fashion (5,18,19) and, therefore, probably interacts with negatively charged side groups related to Na^+ binding and/or translocation. Protection of the antiporter also by ß-adrenergic antagonists indicates that these ligands bind to the Na^+/H^+ exchanger and thereby prevent its reaction with DCCD. Binding and protection could occur at the Na^+ site. Alternatively, ß-antagonists could bind to another part of the antiporter molecule resulting in a conformational change which precludes interaction with DCCD.

Propranolol decreases also the incorporation of [^{14}C]DCCD into rat renal brush-border proteins migrating in SDS gels as a broad band around 65 kDa. The decrease in labeling of only this band depends on propranolol concentration and is incomplete even at maximal concentrations. Qualitatively and quantitatively, this behaviour compares well with the protection of the Na^+/H^+ exchanger from irreversible inactivation. Since in earlier studies (5) amiloride protected the same 65 kDa band from labeling with [^{14}C]DCCD, proteins in this M_r range interact with DCCD, amiloride and propranolol and, thus, may be related to the renal Na^+/H^+ exchanger. Calculations taking into consideration the maximal protection by propranolol (~1000 dpm/band), the specific activity of [^{14}C]DCCD ($1.2 \cdot 10^{14}$ dpm/mol), and the fraction of Na^+/H^+ exchangers modified by 5 nmol DCCD/mg protein (~3%; 50% is modified at 190 nmol DCCD/mg protein; ref. 5) yield that ~4% of the total membrane protein bind [^{14}C]DCCD in a propranolol-protectable manner. Whether the Na^+/H^+ exchanger comprises that much of the total membrane proteins needs to be established.

A final comment relates to the comparison of substrate specificities of adrenergic receptors and the renal Na^+/H^+ exchanger. Inhibition of the exchanger by the α_2 antagonist, clonidine (6), and the ß-antagonists, alprenolol and propranolol, clearly do not reflect the specificity of either α- or ß-adrenergic receptors. Nevertheless, the data indicate that adrenergic receptors and Na^+/H^+ exchanger share some ligands which suggests some common physico-chemical properties of the ligand binding site. It will be an interesting task to i) determine the location of the ligand binding sites within receptor and antiporter molecules and ii) to compare the molecular features of these sites. A closer knowledge of these sites could provide the basis for developing probes and inhibitors of high specificity and affinity.

ACKNOWLEDGMENT

This study was supported by the Deutsche Forschungsgemeinschaft, SFB 169/A2.

REFERENCES

1. Murer, H., Hopfer, U., and Kinne, R. (1976) Biochem. J. 154, 597-604
2. Alpern, R. J. (1990) Physiol. Rev. 70, 79-114
3. Burckhardt, G., and Warnock, D. G. (1990) Semin. Nephrol. 10, 93-103
4. Kleyman, T. R., and Cragoe, E. J. jr. (1988) J. Membrane Biol. 105, 1-21
5. Friedrich, T., Sablotni, J., and Burckhardt, G. (1986) J. Membrane Biol. 94, 253-266
6. Kulanthaivel, P., Leibach, F. H., Mahesh, V. B., Cragoe, E. J. jr., and Ganapathy, V. (1990) J. Biol. Chem. 265, 1249-1252
7. Ganapathy, M. E., Leibach, F. H., Mahesh, V. B., Devoe, L. D., and Ganapathy, V. (1986) Biochem. Pharmacol. 35, 3989-3994
8. Ganapathy, V., Balkovetz, D. F., Miyamoto, Y., Ganapathy, M. E., Mahesh, V. B., Devoe, L. D., and Leibach, F. H. (1986) J. Pharmacol. Exp. Ther. 239, 192-197
9. Frelin, C., Vigne, P., Barbry, P., and Lazdunski, M. (1986) Eur. J. Biochem. 154, 241-245
10. Cantiello, H. F., and Lanier, S. M. (1989) J. Biol. Chem. 264, 16000-16007
11. Bhalla, R. C., and Sharma, R. V. (1986) J. Cardiovasc. Pharmacol. 8, 927-932
12. Häussinger, D., Brodde, O.-E., and Starke, K. (1987) Biochem. Pharmacol. 36, 3509-3515
13. Howard, M. J., Mullen, M. D., and Insel, P. A. (1987) Am. J. Physiol. 253, F21-F25
14. Nunnari, J. M., Repaske, M. G., Brandon, S., Cragoe, E. J. jr., and Limbird, L. E. (1987) J. Biol. Chem. 262, 12387-12392
15. Guyer, C. A., Horstman, D. A., Wilson, A. L., Clark, J. D., Cragoe, E. J. jr., and Limbird, L. E. (1990) J. Biol. Chem. 265, 17307-17317
16. Wilson, A. L., Guyer, C. A., Cragoe, E. J. jr., and Limbird, L. E. (1990) J. Biol. Chem. 265, 17318-17322
17. Biber, J., Stieger, B., Haase, W., and Murer, H. (1981) Biochim. Biophys. Acta 647, 169-176
18. Igarashi, P., and Aronson, P. S. (1987) J. Biol. Chem. 262, 860-868
19. Kinsella, J. L., Wehrle, J., Wilkins, N., and Sacktor, B. (1987) J. Biol. Chem. 262, 7092-7097