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The Effect of Tienilic Acid on Na^+ and K^+ Transport in Human Red Cells

DANIELE CUSI[1] AND RICARDO GARAY

Institut National de la Santé et de la Recherche Médical U7, Centre National de le Recherche Scientifique LA 318, Department of Nephrology, Hôpital Necker, 75015 Paris, France

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SUMMARY

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Tienilic acid inhibits erythrocyte Na^+,K^+ cotransport to a lesser extent than does furosemide. This property seems to be proportional to its diuretic effects. Under conditions in which the erythrocytes have all of their saturable Na^+ and K^+ transport systems blocked, the addition of tienilic acid induces an increase in K^+ permeability. This effect shows saturation kinetics with the increase in the internal K^+ concentration and could not be inhibited by ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid quinine, carbocyanine, or tetraethylammonium. These results suggest that the main effects of tienilic acid on transmembrane cation transport may be the opening of transient or permanent K^+ channels.

INTRODUCTION

The use of pharmacologically active drugs has permitted the characterization of the different Na^+ and K^+ transport systems in human erythrocytes. Ouabain inhibition of an exchange of intracellular Na^+ by extracellular K^+, tightly coupled to ATP hydrolysis, led to the characterization of the Na^+, K^+ pump (1–3). The inhibition of a simultaneous influx (4, 5) or efflux (6) of both Na^+ and K^+ by furosemide led to the characterization of the Na^+, K^+ cotransport system. In addition, the use of phloretin resulted in characterization of the 1:1 Na^+:Na^+ exchange (7–9), and the use of 4,4′-diisothiocyanostilbene-2,2′-disulfonate (10) showed the existence of Na^+ transport as $NaCO_3$ by the furosemide-sensitive anion carrier (11). Moreover, the residual Na^+ and K^+ transport insensitive to all of these drugs has the properties of a nonspecific ground membrane "leak" for monovalent cations (6, 12). Finally, the increase in intracellular Ca^{2+} above physiological levels opens a K^+ channel (13) which is blocked by quinine (14) and carbocyanine (15) ("Gardos effect").

The considerable advance in the knowledge of the molecular mechanisms of ionic transport and the development of simple and precise methods for measure Na^+ and K^+ fluxes in human red cells will in turn permit a better understanding of the molecular pharmacology of cation transport. When the effect of diuretic drugs on Na^+ and K^+ transport in human red cells was analyzed it was found that tienilic acid, an ethacrynic-like molecule, increased passive K^+ permeability. A kinetic analysis of this phenomenon is described herein.

METHODS

Freshly drawn venous blood (15–20 ml) from healthy donors, collected in heparinized tubes, was centrifuged at 1750 × *g* for 10 min and the plasma and buffy coat were removed. The red cells were washed twice with approximately 10 volumes of 150 mM sodium chloride and recentrifuged for 3 min at 1750 × *g*. All steps were carried out at 4°.

The red cells were loaded with Na^+ and choline and depleted of internal K^+, using a slight modification of a procedure which has been described previously (16). Five milliliters of washed, packed cells were suspended in a Na^+/choline-loading medium to a final hematocrit of 8%. The Na^+/choline medium contained 50 mM sodium chloride, 3 mM potassium chloride, 200 choline chloride, 2.5 mM sodium phosphate (pH 7.2 at 4°), 1 mM magnesium chloride, 1 mM EGTA[2], and 0.02 mM PCMBS. This medium is hypertonic since in the presence of PCMBS the loss of K^+ from the cell is more rapid than the gain of choline. Under these conditions, the cell volume does not change after PCMBS treatment. Cells were incubated, with mild agitation, for 20 hr at 4° in Na^+/choline-loading medium, which was renewed after the first 6 hr

[1] Permanent address, Ist Clinica Medica I, Lab. Ipertensione, Osp. Policlinico, 20100 Milano, Italy.

[2] The abbreviations used are: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; PCMBS, 2,5-*p*-chloromercurbenzenesulfonate.

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of incubation. Cells were then centrifuged at 1750 × *g* at 4° for 10 min and the supernatant was discarded. Cells were then resuspended in a "recovering medium" containing 150 mM sodium chloride, 1 mM magnesium chloride, 5.4 mM sodium phosphate (pH 7.2 at 37°), 4 mM cysteine, 2 mM adenine, 3 mM inosine, and 10 mM glucose to give a final hematocrit of 10%. The pH of the recovering solution was adjusted to 7.2 at 37° with Tris base. Cell suspensions were incubated at 37° for 1 hr. Cells were then centrifuged at 4° for 3 min at 1750 × *g*, washed six times with a cold solution of 110 mM magnesium chloride, and resuspended in the same solution to obtain a hematocrit of approximately 60%. An aliquot of this suspension together with an aliquot of cells untreated with PCMBS were set aside to measure intracellular Na^+, K^+, hemoglobin, and hematocrit. The hemoglobin content per liter of PCMBS-treated cells was much the same as that from untreated cells, suggesting no change in cell volume secondary to PCMBS treatment.

Measurement of cation movements. A portion (1 ml) of the cell suspension was added to three tubes containing 15 ml of cold magnesium-sucrose medium with (a) 2 mM KCl (K^+ medium); (b) 0.1 mM ouabain (ouabain medium), and (c) 0.1 mM ouabain plus 1 mM furosemide (furosemide medium). The magnesium-sucrose medium contained 75 mM magnesium chloride, 85 mM sucrose, 5 mM glucose, and 10 mM morpholinopropanesulfonic acid-Tris (pH 7.2 at 37°). The resulting suspensions were pipetted into six tubes (2.5 ml/tube) and incubated at 37° with continuous agitation. Two tubes containing cells incubated in K^+ medium were removed every 15 min (at 0.25, 0.5, and 0.75 hr), and two tubes containing cells incubated in either ouabain medium or furosemide medium were removed every 30 min (at 0.5, 1, and 1.5 hr). The tubes were transferred to 0° for 1 min and then centrifuged at 4° for 3 min at 1750 × *g*. The supernatant was carefully removed (avoiding pellet contamination). Na^+ and K^+ contents were measured by flame photometry (in the K^+ medium, only Na^+ was measured). In control experiments no evidence of red cell lysis during the incubation in the efflux medium could be detected. A small contamination with Na^+ and K^+ due to slight initial hemolysis (at time zero) was subtracted from all of the tubes (see ref. 6 for details).

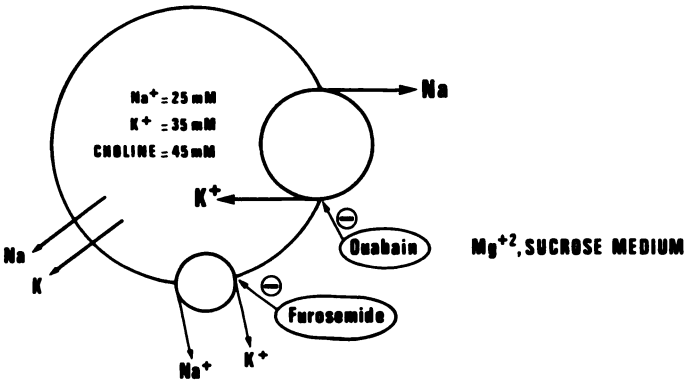

FIG. 1. *Na^+ and K^+ transport across human red cell membranes in Mg^{2+}-sucrose medium containing K^+*

Ouabain blocks the exchange of internal Na^+ for external K^+ catalyzed by the Na^+,K^+ pump. Furosemide blocks the 1:1 Na^+: K^+ efflux catalyzed by the Na^+,K^+ cotransport system. The ouabain- and furosemide-resistant Na^+ and K^+ efflux represent the ground membrane "leaks" for monovalent cations.

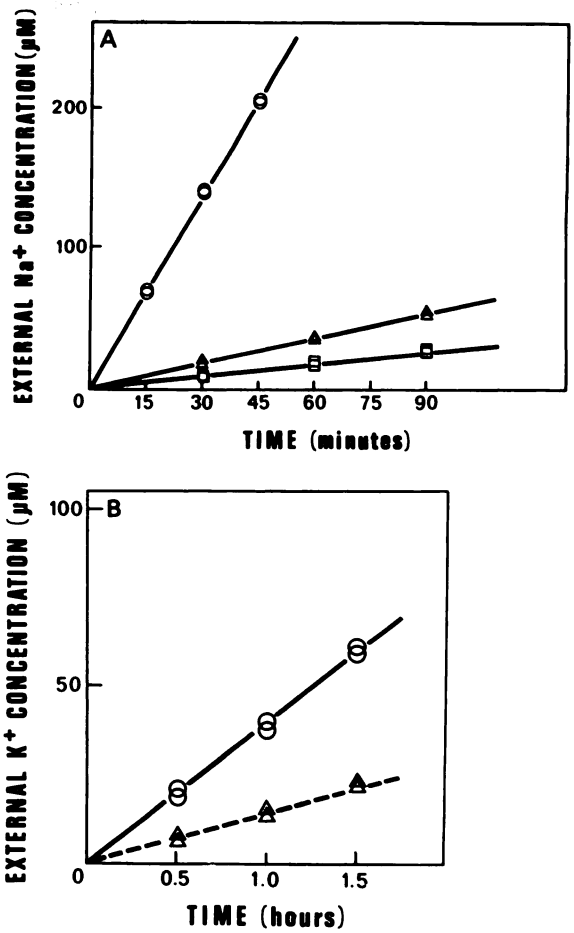

FIG. 2. *Na^+ and K^+ efflux from human erythrocytes into Mg^{2+}-sucrose-medium*

A. The effect of external K^+, ouabain, and furosemide on the Na^+ efflux from human erythrocytes into a Mg^{2+}-sucrose medium. ○, K^+ medium; △, ouabain medium; □, furosemide medium.

B. The effect of furosemide on the K^+ efflux from human erythrocytes into a Mg^{2+}-sucrose medium. ○, ouabain medium; △, furosemide medium.

Calculation of Na^+ and K^+ efflux. Data relating extracellular Na^+ and K^+ concentrations as a function of time (Fig. 2) were subjected to linear regression analysis. The efflux rate in mmoles per liter of cells per hour was obtained by dividing the regression slope by the final hematocrit.[3] The ouabain-sensitive Na^+ efflux was obtained by subtracting the efflux value in ouabain medium from that in K^+ medium. The furosemide-sensitive Na^+ and K^+ efflux was obtained by subtracting the efflux value in furosemide medium from that in ouabain medium.

The effect of drugs. The action of several antihyper-

[3] This method introduces a systematic error of about 4% given that the exact formula for the calculation of fluxes is

$$\text{Flux} = \text{slope} \times \frac{(1 - \text{hematocrit})}{\text{hematocrit}}$$

tensive drugs with diuretic properties was studied in human erythrocytes using the protocol described above. The efflux of Na^+ and K^+ from human erythrocytes into the K^+, ouabain, and furosemide media was studied with and without 1 mM concentrations of the different antihypertensive drugs added to the medium. The drugs were dissolved in a minimal amount of water, ethanol, or dimethyl sulfoxide provided that the final concentration of these solvents had no effect per se on Na^+ and K^+ transport.

RESULTS

Internal cation composition and cell volume of the red cells. After the PCMBS treatment, the cells contained 25–35 mmoles of Na^+ and K^+ per liter of cells. The isotonicity and normal volume of the cells was maintained with the incorporation into the cells of 45–55 mmoles of choline per liter of cells. Under these conditions the internal sites of the Na^+,K^+ pump (17) and of the Na^+,K^+ cotransport (6) are saturated with the relevant cations. The assay of the Na^+,K^+ pump was performed in a 2 mM KCl solution (Fig. 1). Thus, the external sites of the pump are also saturated with external K^+ (1, 2). On the other hand, the furosemide-sensitive Na^+ and K^+ fluxes from the erythrocytes saturated with internal Na^+ and K^+ were measured in a Na^+/K^+-free solution and thus correspond to the maximal rate of outward cation translocation by the Na^+,K^+ cotransport system.

A simultaneous assay of the Na^+,K^+ pump, Na^+,K^+ cotransport, and passive Na^+ and K^+ permeabilities. The release of Na^+ and K^+ from the "saturated" erythrocytes into a Mg^{+2}-sucrose medium is the result of the simultaneous transport by different pathways (Fig. 1). Both cations are released by the furosemide-sensitive Na^+,K^+ cotransport and by the passive Na^+ and K^+ permeabilities. The addition of external K^+ induces a Na^+ efflux catalyzed by the ouabain-sensitive N^+,K^+ pump. Thus, measurements of Na^+ and K^+ efflux from human erythrocytes into a Mg^{+2}-sucrose medium containing KCl, ouabain, and/or furosemide permit the simultaneous assay of three Na^+ (Fig. 2A) and two K^+ (Fig. 2B) transport pathways simply and without the need to use isotopes. Control experiments confirm that the flux rates of these transport pathways are not modified by PCMBS treatment (6, 16).

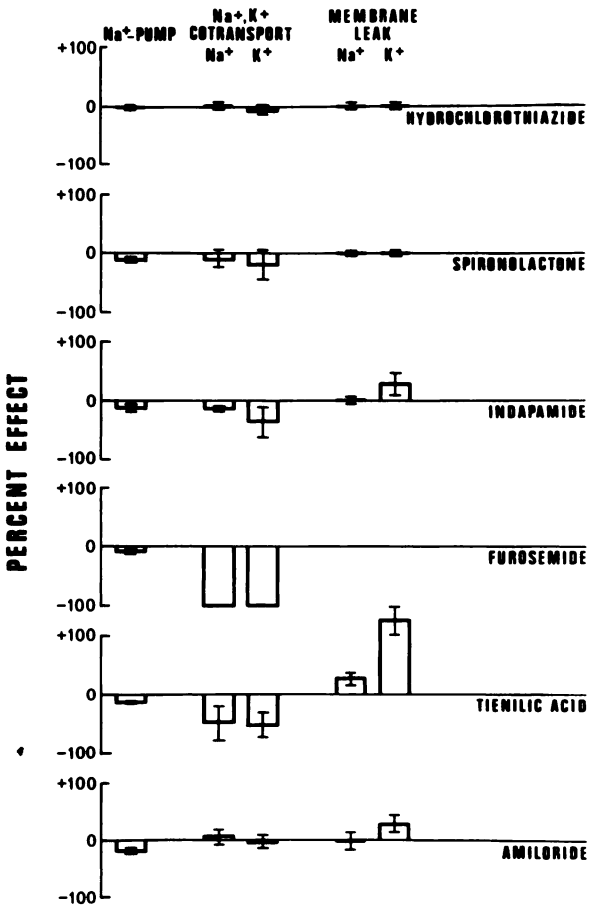

FIG. 3. *The effect of antihypertensive drugs with diuretic properties on Na^+ and K^+ transport in human red cells*

Stimulation of +100% indicates two times normal; −100% indicates 100% inhibition. The bars indicate standard deviations of three to seven experiments.

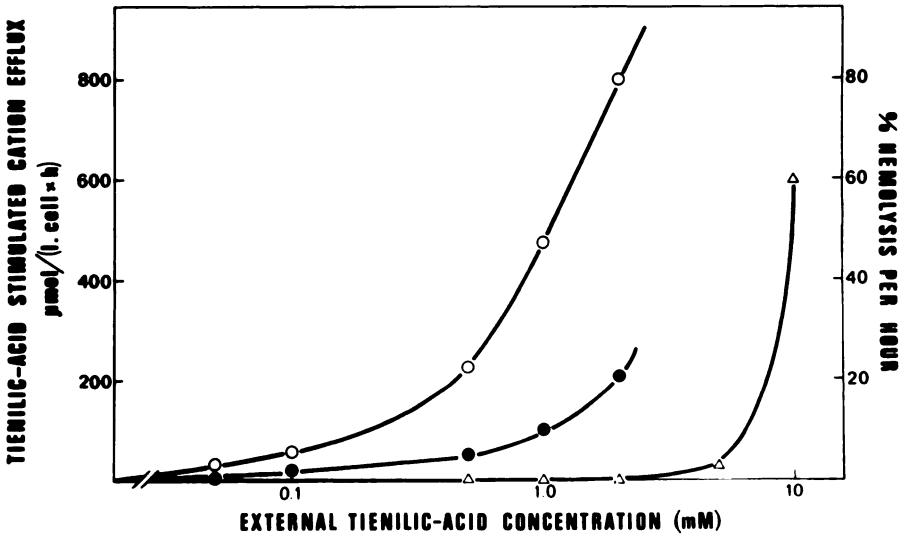

FIG. 4. *The effect of tienilic acid on the ouabain- and furosemide-resistant Na^+ (●) and K^+ (○) efflux into a Mg^{2+}-sucrose medium*

After the loading with Na^+ and choline, no change in cell volume was observed and the cells contained 22 and 44 mmoles of Na^+ and K^+ per liter of cells, respectively. △, The percentage of hemolysis.

This simple transport assay allows us to test the effect of several diuretics on Na^+ and K^+ transport in human red cells. Figure 3 shows that most of these diuretics are slight inhibitors of the Na^+,K^+ pump and more powerful inhibitors of Na^+,K^+ cotransport (in particular furosemide). In addition, some of these diuretics, and particularly tienilic acid, greatly enhance the passive K^+ permeability with little effect on the passive Na^+ permeability.

The inhibitory effect of furosemide on Na^+,K^+ cotransport has been extensively studied elsewhere (5, 6, 16).

The effect of tienilic acid on the passive K^+ permeability. Human red cells incubated in a Mg^{+2}-sucrose medium containing ouabain and furosemide have all of their saturable Na^+ and K^+ transport systems blocked or silent (6, 16). Under these "ground" conditions, the loss of intracellular Na^+ and K^+ can be mediated only by passive leaks (see Fig. 1). The addition of tienilic acid to fresh or PCMBS-treated erythrocytes incubated under these ground conditions induces an almost selective increase in the K^+ efflux (Fig. 4). A dose-response curve shows that this effect increases abruptly with the increase in the tienilic acid concentration (Fig. 4). Higher concentrations of tienilic acid enhance the Na^+ efflux and cause extensive hemolysis. No significant hemolysis could be detected at tienilic acid concentrations of less than 2 mM, suggesting an all-or-none response.

The kinetic properties of the tienilic acid-dependent increase in passive K^+ permeability. In the presence of tienilic acid, the K^+ efflux as a function of the internal K^+ concentration shows two components: (a) a linear component corresponding to the ground K^+ leak and (b) a saturable component corresponding to the fraction of K^+ efflux stimulated by tienilic acid (Fig. 5A). This saturable component cannot be fitted by a simple hyperbolic function but appears to be a sigmoidal (S-shaped) function of the internal K^+ concentration with a 50% activation (K_{50}) at 30–40 mmoles/liter of cells and a maximal rate of efflux of about 700 μmole/liter of cells per hour (Fig. 5B). The shape of this curve is independent of the internal Na^+ concentration, suggesting a high K^+ specificity of the effect. These kinetic properties clearly show that the above effect of tienilic acid is not a simple increase of the ground K^+ leak. They suggest the opening of a discrete number of potassium channels with low Na^+ affinity.

Tienilic acid also enhances the ouabain- and furosemide-resistant K^+ influx (Fig. 6). This effect is independent of the external Na^+ concentration and is linearly dependent on the external K^+ concentration. This would imply that any tienilic acid-dependent potassium channels have low affinity for both external Na and K.

The action of inhibitors of K^+ channels. In excitable cells, selective cation channels are involved in action potentials (18). However, other, nonexcitable, cells also have selective cation channels such as the Ca^{+2}-dependent K^+ channels responsible for the Gardos effect in human red cells (13).

EGTA, quinine, or carbocyanine, which are well known inhibitors of the Gardos effect (14, 15), cannot suppress the effect of tienilic acid on K^+ transport (Fig. 7). In addition, tetraethylammonium, which blocks some K^+ channels in excitable cells, is also unable to inhibit the effect of tienilic acid. These results suggest that the tienilic acid-dependent K^+ channels are different from those mediating the Gardos effect or those sensitive to tetraethylammonium in excitable cells.

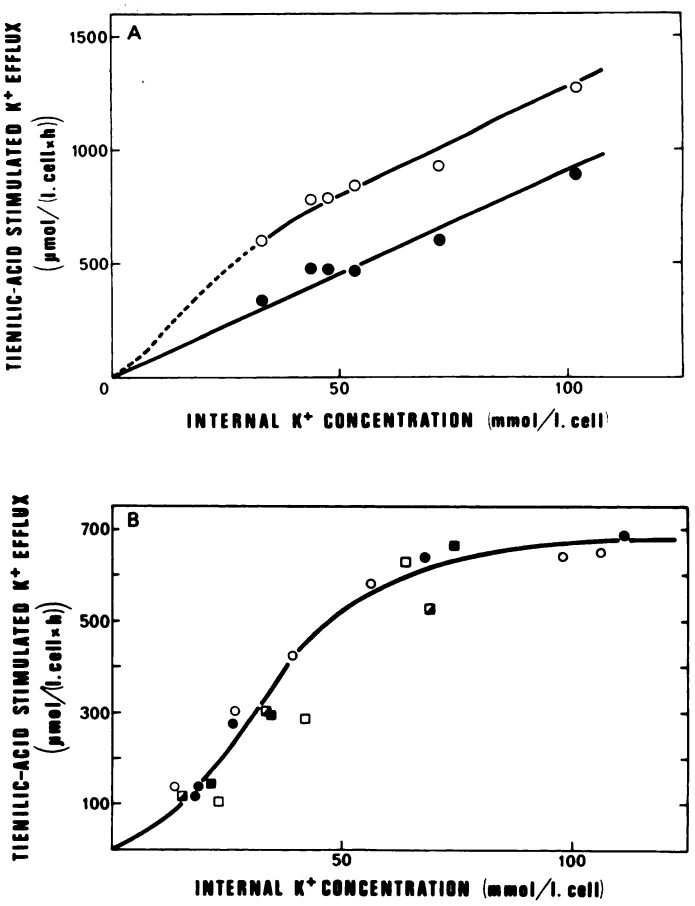

FIG. 5. *K^+ efflux*

A. The ouabain- and furosemide-resistant K^+ efflux in the presence (○) and absence (●) of 0.7 mM tienilic acid as a function of the internal K^+ concentration. In this experiment, K^+ replaces internal Na^+ (see ref. 6 for details on methods).

B. Tienilic acid-stimulated K^+ efflux as a function of the internal K^+ and Na^+ concentration. In two experiments (○, ●) internal K^+ was replaced by Na^+. In a third experiment internal Na^+ was maintained constant at 4.5 (□), 30 (◪), and 54–96 (■) mmoles/liter of cells. Isotonicity was maintained with choline. The external concentration of tienilic acid was 1 mM. The experimental points appears to be fitted by a sigmoidal (S-shaped) curve with a 50% stimulation at 30–40 mmoles/liter of cells of internal K.

DISCUSSION

Tienilic acid is a diuretic and uricosuric drug which has a structure similar to that of ethacrynic acid but which is not an —SH reagent (19, 20). Its antihypertensive properties cannot be accounted for entirely by its natriuretic effect, but also involve inhibition of the membrane excitability of vascular smooth muscle cells (21).

The human red cell is not the target cell for antihypertensive drugs but is one of the best systems for studying Na^+ and K^+ transport at the molecular level. We decided to investigate whether the diuretic and vascular effects of tienilic acid involve the Na^+ and K^+ transport systems present in red cells and further to elucidate their molecular mechanisms.

After a single oral dose of 250 mg, the tienilic acid

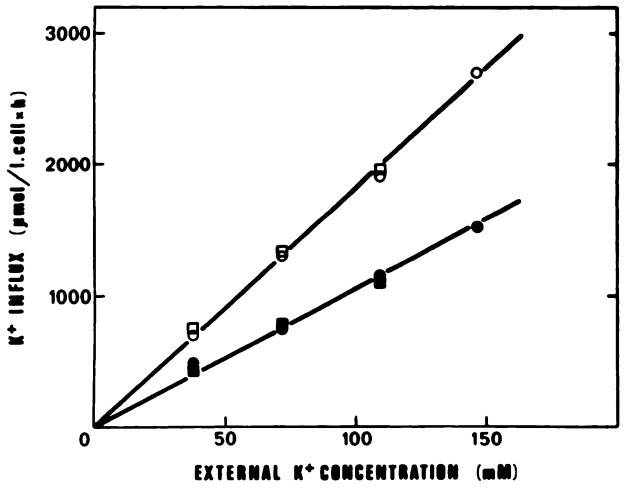

FIG. 6. *The effect of tienilic acid on the ouabain- and furosemide-resistant K^+ influx*

External K^+ was replaced with Na^+ (□) or with choline (○). ●, ■, K^+ influx in the presence of 1 mM tienilic acid. Cells were depleted of internal Na^+ and K^+ and loaded with choline using the PCMBS procedure. The internal K^+ concentration was 5 mmoles/liter of cells.

concentration in the plasma reached a peak of about 0.1 mM (with 95% of the drug bound to plasma proteins; ref. 20). It therefore seems improbable (Fig. 4) that any possible side effects seen after tienilic acid administration should be due to hemolysis (Fig. 4). Tienilic acid is filtered and excreted into the proximal tubule of the nephron. Since the renal clearance of tienilic acid is close to that of inulin, it seems likely that this drug is concentrated in the thick ascending limb of the loop of Henle and in the distal tubule to reach concentrations higher than those used in our experiments with red cells. The thick ascending limb of the loop of Henle is an important site of action for the more potent diuretics such as ethacrynic acid and furosemide (22, 23). In this part of the nephron Na^+ is first reabsorbed across the luminal border of the cells by a furosemide-sensitive Na^+,Cl^-/ cotransport (24, 25). Interestingly, this system shows

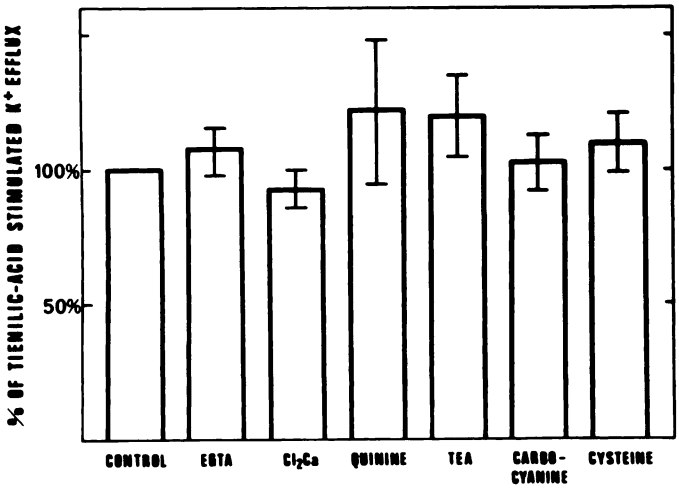

FIG. 7. *The effect of agents active on K^+ channels on the tienilic acid-stimulated K^+ efflux*

All drugs were used at 1 mM with the exception of carbocyanine (0.3 μM). The bars indicate standard deviations of three to five experiments.

many similarities with the furosemide-sensitive Na^+,K^+ cotransport of human red cells (4–6, 16, 26, 27), suggesting that both modalities of transport could be partial reactions of a same furosemide-sensitive Na^+,K^+,Cl^- cotransport as proposed for avian red cells (28). Figure 3 shows that tienilic acid is a less efficient inhibitor of erythrocyte Na^+,K^+ cotransport than furosemide as well as being a weaker diuretic. In addition, the thiazides and K^+-sparing diuretics, which seem to act by a different mechanism, do not inhibit the Na^+,K^+-cotransport system (Fig. 3). Thus, the simple assay of the erythrocyte Na^+,K^+ cotransport may be a useful diuretic test for furosemide or ethacrynic-like drugs.

The injection of ouabain into the renal artery of dogs induces a prompt and significant diuresis (29). This and other related observations led to the proposal that the inhibition of the Na^+,K^+ pump was the common mechanism of action of diuretic drugs. However, Fig. 3 shows that, even at doses (1 mM) never attained in the plasma of treated patients, all of the diuretics studied were unable to inhibit more than 20% of pump fluxes. This negative result does not exclude a pump inhibition mechanism from the cytoplasmic aspect of renal cell plasma membranes.

In addition to a furosemide-like inhibition of the erythrocyte Na^+,K^+ cotransport, tienilic acid dramatically increases the passive K^+ permeability of the red cells. The fact that this effect is almost selective for K^+ and that it shows saturation with increasing internal K^+ concentration excludes the possibility of a simple increase in the ground membrane leak for monovalent cations.[4] Moreover, since the tienilic acid increase in K^+ efflux is smaller than the Gardos effect and cannot be inhibited by EGTA, quinine, or carbocyanine, it is most likely that tienilic acid opens a discrete number of "transient" or permanent K^+ channels, different from those involved in the Gardos effect. The distinction between transient and permanent K^+ channels is important because a permanent channel corresponds to a genetically coded transport system and a transitory channel corresponds to discrete and transient structural "defects" of the membrane bilayer which, under physiological conditions, are unlikely.[5] Organic anions such as dinitrophenolate, picrate, and trinitrocresoleate also cause an increase in K^+ and Na^+ leaks, possibly by forming lipid-soluble ion pairs with Na^+ and K^+ (30). Such a mechanism cannot be excluded for tienilic acid.

In conclusion, our results indicate that, in addition to a slight diuretic-like inhibition of the Na^+,K^+ cotransport, tienilic acid opens a K^+ channel in human red cells. Such an effect in vascular smooth muscle cells may inhibit basal arterial tone by membrane hyperpolarization.

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[4] We cannot rule out the possibility of a discrete and 'reversible' lysis as observed in vesicles, which is accompanied by pseudo-carrier mediated fluxes (V. Lew, personal communication).

[5] V. Lew, personal communication.

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Send reprint requests to: Dr. R. Garay, INSERM U7, CNRS LA 318, Department of Nephrology, Hôpital Necker, 161 rue de Sévres, 75015 Paris, France.