Effects of Fluoride on Potassium and Sodium Permeability of the Erythrocyte Membrane

SIGRID LEPKE and H. PASSOW

From the Department of Radiation Biology, The University of Rochester, Rochester, New York 14621, and the Second Department of Physiology, The University of Saarland, Homburg/Saar, Germany

One of the most important features of cell membranes is their capacity to discriminate between ions of different sign or different chemical nature. In the erythrocyte membrane, coulombic forces seem to govern the discrimination between anions and cations. There is reason to believe that, without impeding the movements of anions, positive fixed charges impose a severe restriction on the movements of diffusible cations between cells and medium. But although a number of very conspicuous changes of cation permeability with pH and ionic strength can be related to variations of the fixed charge density of the membrane (Passow, 1965; LaCelle and Rothstein, 1966), observations have been made that passive cation permeability is not entirely governed by coulombic forces. In particular, it has been observed that a number of agents such as fluoride, lead, triose reductone, IAA and adenosine (Passow, 1964), and others are capable of inducing a selective increase in passive K^+ movements without greatly affecting Na^+ permeability. It is far from being clear whether these agents act on a physiologically operating cation control mechanism or open up channels which are normally without any control function. Nevertheless, the selectivity of the effect with respect to K permeability raises the generally interesting question about the nature of K/Na discrimination in biological membranes. The present article recapitulates part of the previous work on fluoride poisoning of erythrocytes and adds new information on the site of action of the poison and on the factors controlling K/Na discrimination in the fluoride-treated membrane.

The action of fluoride on washed human erythrocytes suspended in isotonic NaCl depends not only on its own concentration but also on the concentrations of alkaline earth ions and glucose in the medium.

With increasing fluoride concentration its effect on passive cation permeability passes through a maximum. Depending on the respective concentrations of alkaline earth ions at the optimal fluoride concentration of 40 mmoles/liter, three different effects on cation permeability can be distinguished.

(*a*) In the complete absence of alkaline earth ions, i.e. in the presence of complexing agents such as EDTA (Gardos, 1958; Lepke and Passow, 1959) or ATP, fluoride inhibits the pump without affecting passive cation permeability. K^+ leaves

and Na^+ enters the cells at about equal rates of a few millimoles per liter of cells per hour.

(*b*) If the concentration of $CaCl_2$ or $MgCl_2$ in the medium is raised to 0.05 or 0.5 mmoles/liter respectively, then, after a lag period, an increase of net K efflux occurs which may exceed 20 times the rate of loss due to the inhibition of the pump. Since little concomitant Na uptake occurs the cells shrink and attain a high osmotic resistance.

(*c*) Further increasing the concentration of alkaline earth ions may reduce the lag period to virtually zero. In addition the nature of the response changes. The mem-

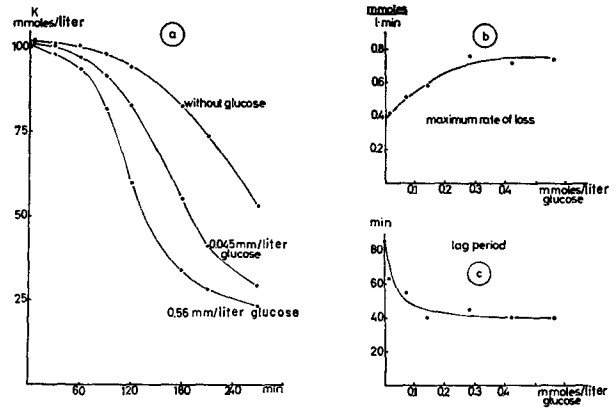

FIGURE 1. Action of glucose on K loss of fluoride-poisoned erythrocytes. Cell concentration 10 volumes/100 volumes; composition of medium, 40 mmoles/liter NaF, 126 mmoles/liter NaCl, 0.05 mmoles/liter $CaCl_2$, and the glucose concentrations indicated on the graph. 37°C. (*a*) Time course of K loss at three representative glucose concentrations. Ordinate, K content of cells in millimoles per liter of original cell volume. Abscissa, time in minutes. (*b*) and (*c*) represent the maximum rate of K loss and the lag period, respectively, as functions of glucose concentrations in the medium. The intersection between a straight line parallel to the abscissa passing through the point representing the initial K content and the straight line used to determine the maximum slope is taken as a somewhat arbitrary but convenient measure for the length of the lag period.

brane now becomes permeable to Na ions which enter the cells at about the same rate at which K ions leave. The cells swell and eventually disintegrate by colloid osmotic hemolysis.

The specific effect on potassium permeability alone (*a*) can be modified by agents which affect the metabolism of the poisoned cells. Addition of substrates, such as adenosine or pyruvate, or oxidizing agents, such as methylene blue or ferricyanide, prevents the specific change of membrane resistence to K ions but exerts little or no influence on the nonspecific damage done by high Ca or Mg concentrations (*c*). It seems reasonable therefore to assume that, depending on the concentration of alkaline earth ions, besides inhibiting the pump, fluoride exerts two different effects on cation permeability. One seems to be related to the capacity of fluoride to inhibit

glycolysis; the other seems to be comparable to those seen in the presence of lysins at sublytic concentrations and to represent an immediate action on the membrane.

Low concentrations of glucose appreciably affect the specific fluoride action on K permeability (*b*) (Whittam, R. Personal communication.): with increasing glucose concentration the lag period shortens and the maximum rate of K loss goes up (Fig. 1). The effective glucose concentrations are too low to account for an indirect action through the formation of glycolytic intermediates. Moreover, glucose exerts similar actions on metabolically inert erythrocyte ghosts. All experiments with ghosts were done therefore in the presence of 10 mmoles/liter glucose.

As has already been stated, certain substrates or intermediates of cell metabolism, e.g., oxidizing agents which act to produce DPN as a substrate for the rate limiting glyceraldehydephosphate dehydrogenase, may suppress the specific action of fluoride; others, like inosine are without influence or may even slightly enhance the rate of K loss. For a time it was believed therefore that fluoride, by blocking enolase, diverted the glycolytic reactions into normally less prominent metabolic pathways and thus

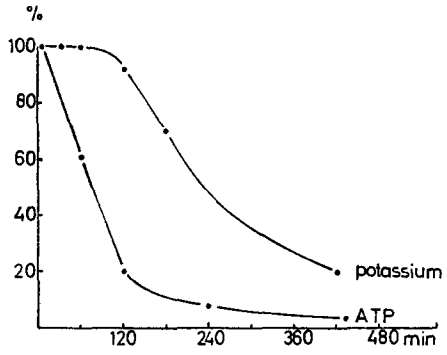

FIGURE 2. Potassium loss (upper curve) and ATP break-down (lower curve) in fluoride-poisoned erythrocytes. Composition of medium, 126 mmoles/liter NaCl, 40 mmoles/liter NaF, 0.5 mmoles/liter $MgCl_2$. Ordinate, K or ATP content in per cent of initial value. Abscissa, time in minutes.

induced, by means of the cell's own enzymes, an increase in concentration of an intermediate which controls the passive potassium permeability (Wilbrandt, 1940; Gardos, 1958). It has been observed however, that substrate depletion, by incubating the cells for many hours in saline solutions prior to the addition of fluoride, augments the sensitivity of the cells to the poison. The lag period shortens (but does not completely disappear) and the rate of K loss increases. Obviously in cells largely devoid of glycolytic intermediates the occurrence of abnormal side reactions is unlikely. Since the permeability change develops only in the presence of traces of alkaline earth ions which penetrate extremely slowly it was suggested that the formation of an alkaline earth fluoride complex with ligands in the outer cell surface of the type L–Me–F is the primary and most important event in fluoride action. In view of the fact that inhibition of glycolysis either by fluoride itself or by substrate deprivation is a prerequisite for the permeability change to occur it was assumed that only after inhibition of glycolysis normally inaccessible surface ligands become susceptible to direct interactions with alkaline earth ions and fluoride.

Fluoride inhibition of glycolysis is associated with ATP breakdown. It may be asked whether removal of that complexing agent is the prerequisite for the fluoride

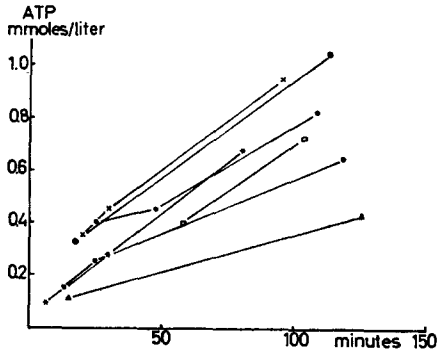

FIGURE 3. Lag period (abscissa) as function of the ATP content at the instant of fluoride poisoning (ordinate). Composition of medium, 40 mmoles/liter NaF, 126 mmoles/liter NaCl, 0.01 mmoles/liter $CaCl_2$, 37°C. Redrawn from Lindemann and Passow, 1960 *b*. Reprinted with copyright permission of Springer-Verlag OHG, Berlin.

action on permeability. Fig. 2 shows that at the end of the lag period the intracellular ATP content has dropped to about 20% of its initial value (see Gardos and Straub, 1957). In cells with varying initial ATP concentrations, as obtained by preincubation in the absence of substrates for various lengths of time, the duration of the lag

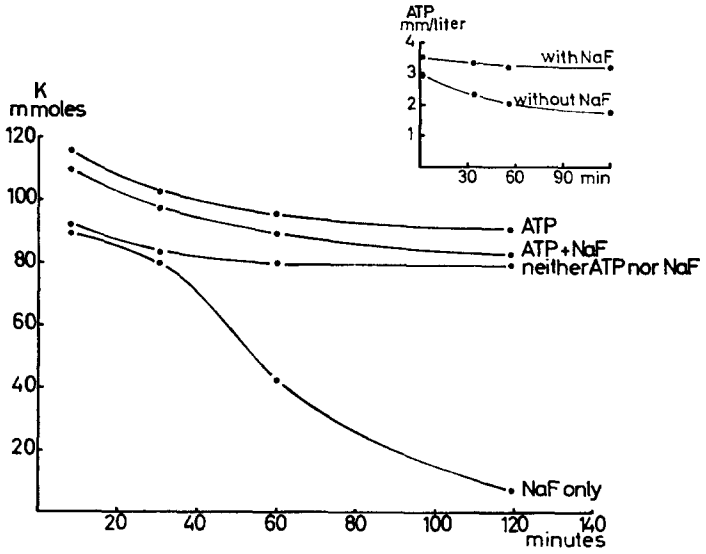

FIGURE 4. Effect of incorporated ATP on potassium loss of fluoride-poisoned erythrocyte ghosts. The ghosts contain 4 mmoles/liter $MgCl_2$ and, where indicated, ATP at about the same concentration (see inset which represents intracellular ATP concentration as function of time). Composition of medium: 0.05 mmoles/liter $CaCl_2$, 126 mmoles/liter NaCl, and where indicated, 40 mmoles/liter NaF. Otherwise the NaF is replaced by NaCl, 37°C. Ordinate, K content per liter of initial ghost volume. Abscissa, time in minutes.

period decreases with decreasing initial ATP content (Fig. 3). Although these findings suggest a participation of ATP in determining the length of the lag period, they give no definite proof, since other equally effective metabolic intermediates may vary in

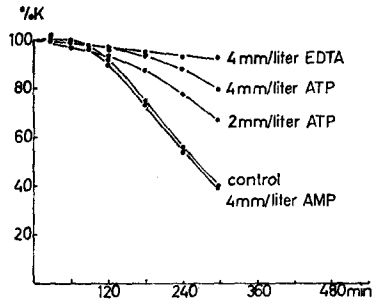

FIGURE 5. Effects of extracellular complexing agents on potassium loss of fluoride-poisoned erythrocytes. Composition of medium: 126 mmoles/liter NaCl, 40 mmoles/liter NaF, 0.05 mmoles/liter $CaCl_2$; no glucose, and complexing agents at the concentrations indicated in the graph, 37°C. Ordinate, K content in per cent of initial value. Abscissa, time in minutes.

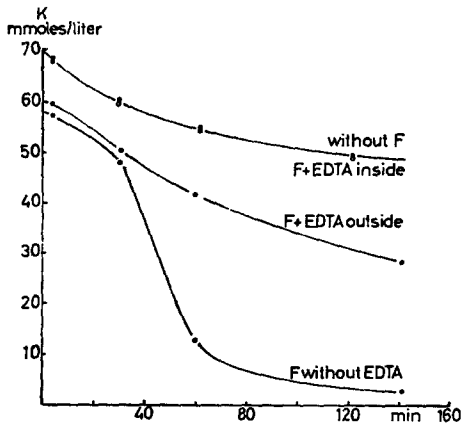

FIGURE 6. K loss of fluoride-poisoned erythrocyte ghosts in the presence of extracellular (F + EDTA outside) or intracellular (F + EDTA inside) EDTA. Two curves are included which show (a) the effect of fluoride in the absence of complexing agents (F without EDTA) and (b) the effect of incorporated EDTA in the absence of fluoride (without F, hollow circles). The ghosts contain 4 mmoles/liter $MgCl_2$ or 4 mmoles/liter EDTA. Composition of medium, 40 mmoles/liter NaF, 126 mmoles/liter NaCl, 0.05 mmoles/liter $CaCl_2$ (without EDTA) or 10 mmoles/liter EDTA (with EDTA).

proportion to the ATP content. Experiments with metabolically inert erythrocyte "ghosts" confirm, however, that fluoride acts directly on the cell membrane and that this action is in fact prevented by ATP (Fig. 4). In the absence of ATP, with ghosts which still contain about 5% of their original cell contents but which were found to be incapable of converting glucose, adenosine, or incorporated fructose diphosphate

into lactate, K loss begins after a lag period of about 20–40 min and thus resembles that observed in substrate-depleted intact cells. The action of ATP is probably associated with its capacity to form chelates with alkaline earth ions. If EDTA instead of ATP is incorporated into the ghosts, fluoride action is nearly as completely prevented as with ATP.

Complexing agents do not only act when applied to the interior of the cells. If present in the medium, EDTA or ATP prevents K loss in spite of the fact that, in intact cells, intracellular ATP is drastically reduced or, in ghosts, is completely absent. In other words, nonpenetrating chelating agents prevent the fluoride action if applied to either the inside or outside of the membrane; they do not need to be present on both sides simultaneously (Figs. 5, 6).

If one accepts the hypothesis that fluoride is bound to the membrane by forming complexes of the type ligand–Me–F it is reasonable to postulate that the chelating agents act either by removing the metal from the membrane or by replacing fluoride.

The fact that in spite of the presence of high concentrations of Mg^{++} inside the ghosts, traces of alkaline earth ions in the medium are required to induce the perme-

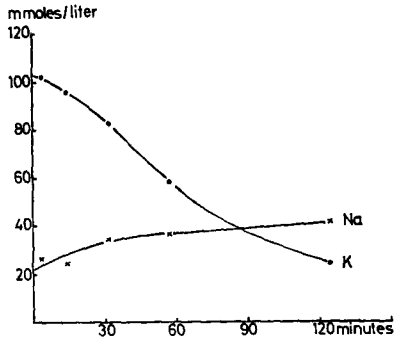

FIGURE 7. Selectivity of fluoride action on cation permeability. K-loaded ghosts suspended in NaCl solution containing 0.05 mmoles/liter $CaCl_2$, 0.25 mmoles/liter glucose, 40 mmoles/liter NaF, 5 mmoles/liter Tris, pH 7.4, temperature 37°C. Ordinate, cation content in millimoles per original cell volume. Abscissa, time in minutes.

ability change suggests that the membrane is barely, if at all, permeable to Mg^{++} or Ca^{++}. It seems unlikely, therefore, that chelating agents applied to one side of the membrane are capable of removing the Me^{++} from the opposite side. Apparently interactions of the chelating agents with the ligands on one membrane surface are sufficient to afford a protection against fluoride or else the fluoride-sensitive sites are accessible to nonpenetrating complexing molecules present within as well as outside the cell.

The most interesting feature of fluoride poisoning, its selectivity with respect to K, is also observed in K-loaded erythrocyte ghosts suspended in isotonic NaCl solution. It appears, therefore, that the nature of the permeability change is similar in ghosts and intact cells and that the ghosts are a useful means for elucidating the nature of K/Na discrimination (Fig. 7).

APPENDIX

The erythrocyte ghosts used in the described experiments were prepared as follows.

Erythrocytes from freshly drawn citrated blood are washed three times in isotonic NaCl and resuspended in isotonic Tris, pH 7.2, for a final concentration of 50 volumes per 100

volumes. This suspension is mixed with 10 times its volume of distilled water containing 4 mmoles/liter $MgSO_4$ and the other substances to be incorporated into the cells, e.g. ATP, etc., except K and Na.

At the instant of osmotic hemolysis a relatively large fraction of extracellular ATP or AMP enters the cells, but diffusion equilibrium is not yet established. The membrane is, however, still leaky for K and Na and even for such large molecules as ATP. So that the ghosts could be filled with K or Na, 5 min after hemolysis concentrated KCl or NaCl, or a mixture of both electrolytes, is added to the medium and thus by a suitable choice of their concentrations the desired intracellular concentrations can be established. In order to reseal the membranes and, hence, to trap incorporated alkali ions and other substances, such as akaline earth ions, ATP, etc., the hemolysate is incubated for 45–60 min at 37°C (Hoffman, Tosteson, Whittam, 1960). The degree of recovery of the membranes is critically dependent on the temperature at which hemolysis took place and is the more complete the lower that temperature was. During posthemolytic incubation equilibration between cells and medium continues. In the case of ATP, only one-third of the final ATP content is taken up during hemolysis at 0°C, but the remaining two-thirds is taken up during the subsequent incubation at 37°C. Even if ATP is added only after hemolysis, a satisfactory ATP incorporation can be achieved.

At the end of the resealing process the ghosts are centrifuged down at 50,000 *g* and washed twice in the desired suspension medium. The rate of net K leakage from such ghosts into isotonic NaCl is somewhat faster than from the intact erythrocytes and amounts to about 3–4 mmoles/liter of ghosts per hour. The instant of resuspension of the ghosts after the second wash is zero time of the experiments presented in the graphs. Sampling was done by pipetting 3.0 ml of the ghosts suspension into 30 ml of a choline chloride solution of the same osmolarity as the cell contents and subsequent centrifugation. The choline chloride solution contained 40 mmoles/liter LiCl. Li is analyzed in the sediment together with Na and K. Comparison with measurements of the sucrose space showed that Li determinations allow a reliable determination of the trapped extracellular fluid.

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