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Modulation of the Ca^{2+}- or Pb^{2+}-activated K^+-selective channels in human red cells. II. Parallelisms to modulation of the activity of a membrane-bound oxidoreductase *

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Modulation of Ca^{2+}-activatable K^+ permeability was compared with modulation of a membrane-bound oxidoreductase activity in human erythrocytes. Changes in the K^+ permeability were monitored by flux measurements and single-channel recordings. The enzyme activity was detected by measuring reduction of ferricyanide. Pb^{2+}, Atebrin and menadione had parallel effects on the channel protein and the enzyme. In contrast, propranolol stimulates K^+ permeability, but is without effect on enzyme activity. The results demonstrate that the K^+ channel and the enzyme are distinct membrane proteins but that the enzyme activity may influence channel gating.

Introduction

Experiments comparing several activators and inhibitors (particularly naphthoquinone derivatives) of a membrane-bound oxidoreductase demonstrated essentially similar effects on Ca^{2+}-activated K^+ permeability in human red cells [1]. These parallel effects led to the suggestion of a possible relationship between the Ca^{2+}-activated K^+ channels and the oxidoreductase. In this paper, the possibility of such a relationship is further investigated. Treatment of red cells that lead to activation or inhibition of oxidoreductase activity also produce parallel effects on the K^+ channels. This effect is also apparent with activation of the K^+ channels by Pb^{2+}. On the other hand, modulation of channel activity does not necessarily result in modulation of oxidoreductase activity. These observations support the view that although the enzyme activity may influence channel activity, the opposite is not the case. Parts of these results have been reported previously [2].

* Dedicated to Prof. Dr. K.J. Netter on the occasion of his 60th birthday.

Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mops, 4-morpholinepropanesulfonic acid.

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Methods

The methods used to determine cation fluxes and single-channel activity were essentially the same as those described in the preceding paper [3]. In the present study, the K^+ channels were activated primarily by Pb^{2+}.

The activity of ferricyanide-NADH dehydrogenase was determined in erythrocyte ghosts treated with or without Triton X-100 and suspended at 37 °C in nitrate medium containing 0.2 mM ferricyanide. After adding 20 μM NADH to start the reaction, reduction of ferricyanide per mg protein was determined as the change in ferricyanide absorbance recorded with a double-beam spectrophotometer at 485 and 420 nm.

To detect transmembrane activity of the enzyme, experiments were performed on intact erythrocytes. Reduction of extracellular ferricyanide by the NADH dehydrogenase was assayed by analysis of ferrocyanide production in the supernatant according to Avron and Shavit [4].

Results

Flux and current measurements

Effects of lead. The dependence of the K^+ permeability on Pb^{2+} concentration has previously been analysed

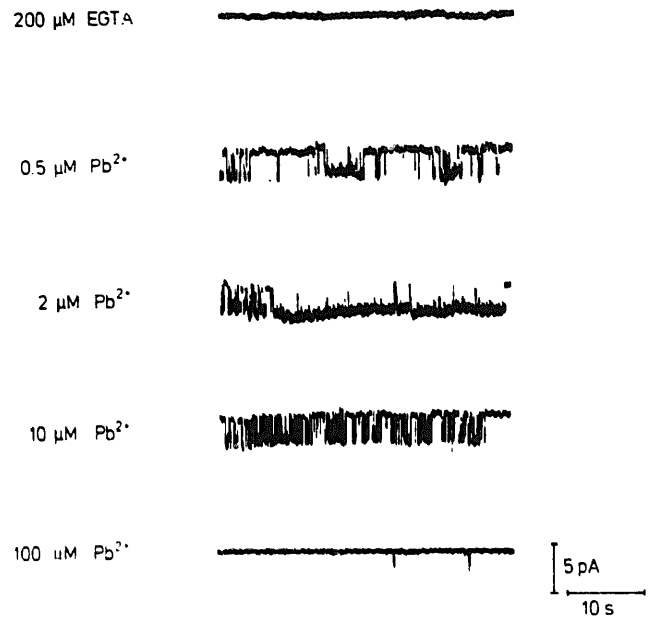

Fig. 1. Effect of different concentrations of $Pb(NO_3)_2$ on the activity of single-channel events measured at a holding potential of −100 mV. Downward deflections reflect inward current through single open channels.

in detail by flux measurements [5]. The effects of various Pb^{2+} concentrations on single-channel events are demonstrated in Fig. 1. Increasing the Pb^{2+} concentration (below 10 μM) activates channel openings in a way similar to Ca^{2+} [6]; this is primarily by increasing mean open times. In contrast to Ca^{2+}, higher concentrations of Pb^{2+} (10 μM or more) drastically inhibit the activity of channel openings. At 100 μM, channel activity is already almost completely inhibited (for flux measurements see Fig. 5). This demonstrates that modulation of the K^+ permeability by Pb^{2+}, as detected in flux measurements, is due to alterations of channel gating, single-channel conductance being unaffected.

Effects of Atebrin. Reduction of the Pb^{2+}-activated K^+ permeability by altered channel gating can also be observed with Atebrin. When single-channel events are elicited by 2 μM Pb^{2+} in inside-out membrane patches, 200 μM Atebrin reduces the probability of channel openings by about 20%, whereas the amplitude of single-channel currents is unaffected (Fig. 2A). A similar degree of inhibition is observed in flux experiments for the Pb^{2+}-activated K^+ permeability (see Fig. 2B). As with higher Pb^{2+} concentrations, this inhibition is

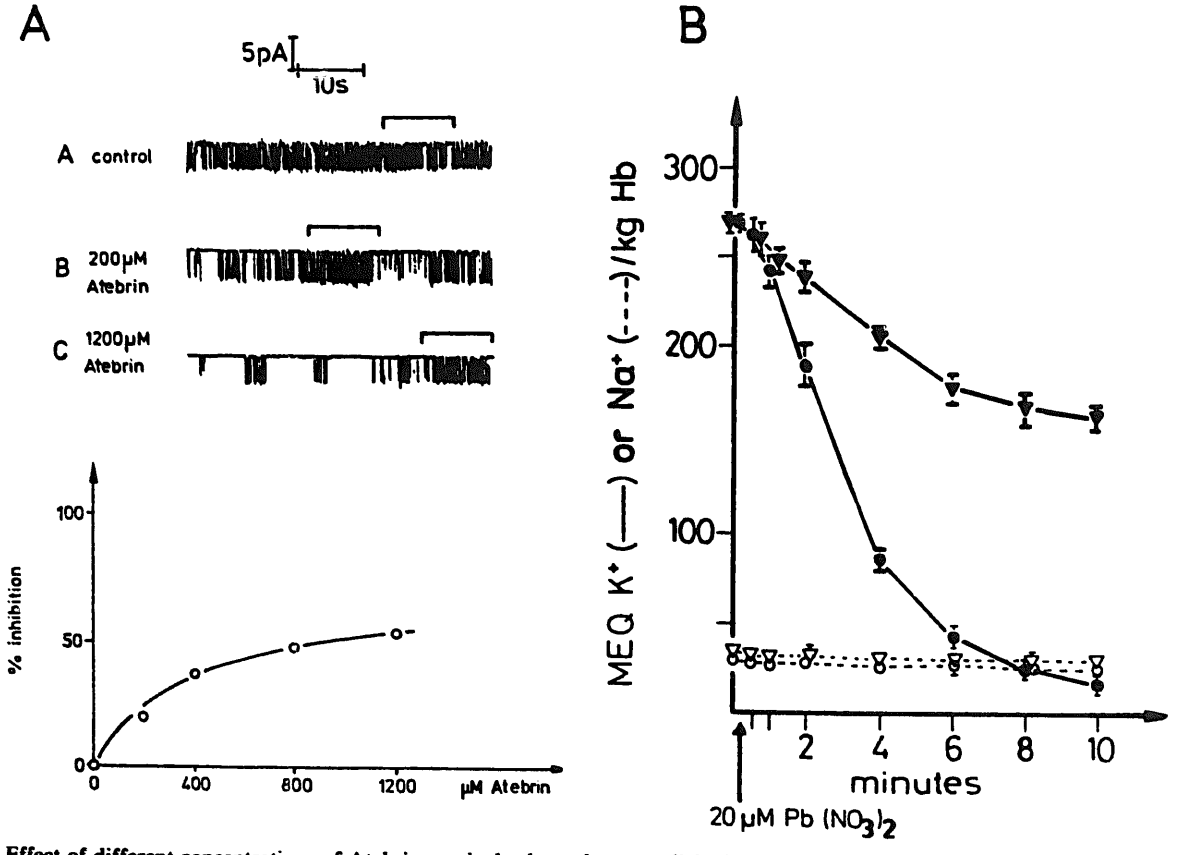

Fig. 2. (A) Effect of different concentrations of Atebrin on single-channel events elicited by 2 μM $Pb(NO_3)_2$ and measured at a holding potential of −100 mV. (Upper part) Downward deflections reflect inward current through single open channels. (Lower part) Dependence of the probability of the open channel state on Atebrin concentrations. (B) Effect of Atebrin on K^+ and Na^+ content of erythrocytes as a function of time. Circles are without, triangles are with 100 μM Atebrin in the incubation medium. The flux experiment was started by adding Pb^{2+} to the medium at the time indicated. Open symbols represent Na^+ content, closed symbols K^+ content; bars indicate S.D. ($n = 4$). MEQ, milliequivalents.

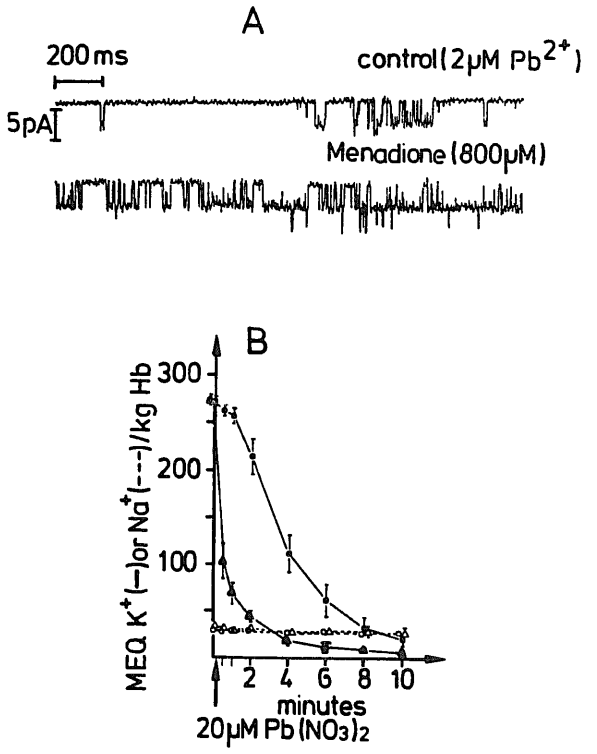

Fig. 3. (A) Effect of menadione on the activity of single-channel events elicited by 2 μM $Pb(NO_3)_2$ and measured at a holding potential of -100 mV. Downward deflections reflect inward current through single open channels. (B) Effect of menadione on K^+ and Na^+ content of erythrocytes as a function of time. Circles are without, triangles with 300 μM menadione in the incubation medium. The flux experiment was started by adding Pb^{2+} to the medium at the time indicated. Open symbols represent Na^+ content, closed symbols K^+ content; bars indicated S.D. ($n = 4$). MEQ, milliequivalents.

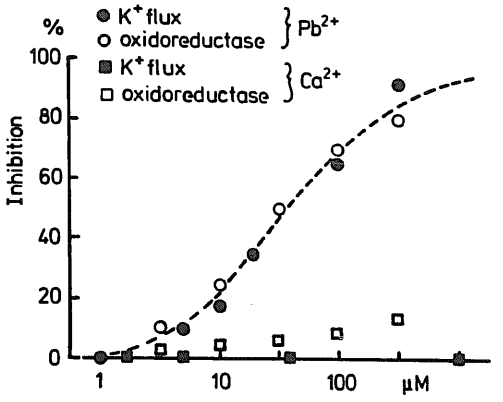

Fig. 5. Dependence of inhibition of K^+ permeability (evaluated from Fig. 4 of Ref. 5) and of oxidoreductase activity on the concentration of Pb^{2+} and Ca^{2+}.

specific for the K^+ permeability; Na^+ fluxes are unaffected.

Effects of menadione. Previously it has been demonstrated that menadione has a stimulating effect on the Ca^{2+}-activated K^+ channels [1]. If Pb^{2+} is used as an activator, menadione also stimulates single-channel activity (Fig. 3) and net fluxes (Fig. 3B). But in metabolically depleted cells ('Gardos-type' conditions [10]) inhibition of K^+ permeability can be observed [1]. It has been suggested that, depending on the metabolic state, menadione can produce either inhibition or activation. To investigate this possibility, further experiments were performed at different times after initiating metabolic depletion by addition of iodoacetic acid and inosine. If no Ca^{2+} is added to the incubation medium, only slight reduction of K^+ content can be detected with time (compare data points in Fig. 4A at 0, 4B at 40, and 4C at 160 min). The slight decline is hardly affected by menadione. If, however, the K^+ channels are activated by adding Pb^{2+} at different times after metabolic deple-

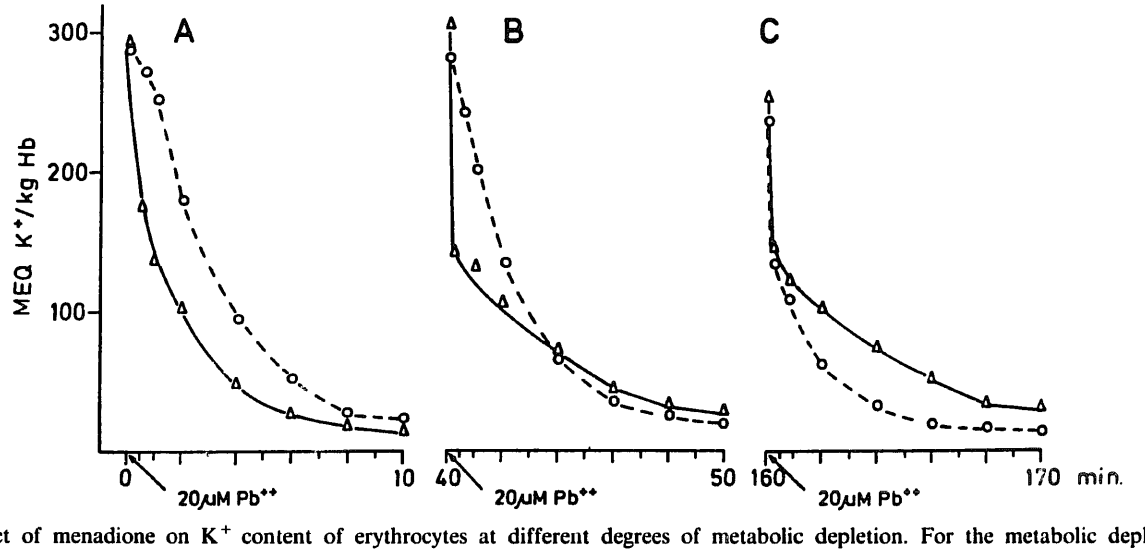

Fig. 4. Effect of menadione on K^+ content of erythrocytes at different degrees of metabolic depletion. For the metabolic depletion, 0.5% erythrocytes were incubated in 150 mM $NaNO_3$ (suprapur), 1 mM KNO_3, 2.5 mM iodoacetate adjusted to pH 7.6 with 100 mM Hepes, 2 mM inosine and without or with 100 μM menadione. Three times (at 0, 40 and 160 min) during incubation, samples were taken and 20 μM $Pb(NO_3)_2$ was added (see arrows). The time course of the subsequent K^+ loss was determined. Circles are without and triangles are with menadione. MEQ, milliequivalents.

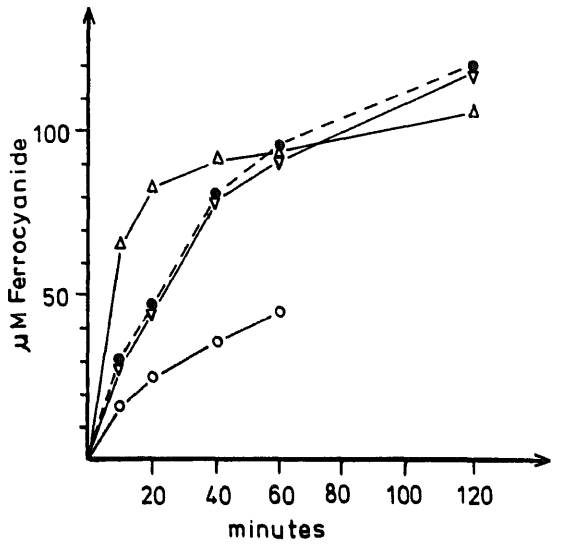

Fig. 6. The production of ferrocyanide by 5% intact erythrocytes suspended in 150 mM NaCl, 10 mM inosine, 0.2 mM $K_3Fe(CN)_6$ adjusted to pH 7.6 by 20 mM Hepes. Experiments were performed at 37 °C. ●——●, control; △——△, 60 µM menadione; ▽——▽, 1 mM propranolol; ○——○, 200 µM $Pb(NO_3)_2$.

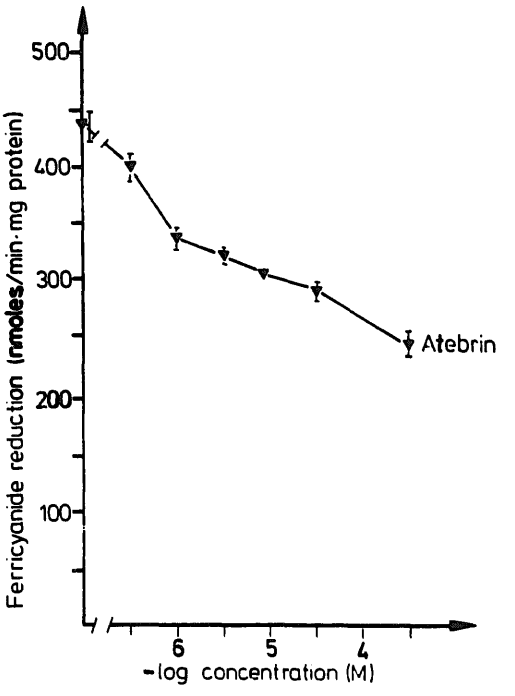

Fig. 7. Dependence of the oxidoreductase activity on the concentration of Atebrin. Symbols represent average values ($n = 3$, ±S.D.).

tion has been initiated, a fast K^+ efflux occurs (Fig. 4A–C). In the presence of menadione, the K^+ efflux is modulated. Before metabolic depletion has started (Fig. 4A), menadione stimulates, as already shown in Fig. 3B. But with increasing metabolic depletion, stimulation changes to inhibition (Fig. 4B, 4C). These results, indeed support the concept that either inhibition or stimulation can occur, depending on the metabolic state of the cell.

Measurements of oxidoreductase activity

Effects of lead. Pb^{2+} concentrations that inhibit K^+ permeability also reduce activity of the ferricyanide-NADH dehydrogenase in permeabilized erythrocyte ghosts [1]. Fig. 5 shows that inhibition of channel activity and of enzyme activity exhibit the same dependence on the Pb^{2+} concentration. These strong inhibitory effects cannot be observed with Ca^{2+}. Fig. 6 demonstrates that Pb^{2+} not only inhibits total dehydrogenase activity in permeabilized ghosts, but also the transmembrane dehydrogenase activity in intact erythrocytes (compare filled and open circles).

Effects of Atebrin. Similarly to higher Pb^{2+} concentrations that inhibit both the K^+ permeability and the activity of the oxidoreductase, Atebrin also reduces oxidoreductase activity (Fig. 7). At an Atebrin concentration of 100 µM, enzyme activity is reduced by about 40%.

Effects of menadione. For ferricyanide-NADH dehydrogenase activity, inhibition by menadione has previously been reported [1]; the corresponding experiments were carried out in the presence of 0.2% Triton X-100. If Triton is omitted, dehydrogenase activity is decreased; menadione increases the activity in a concentration-dependent manner (Fig. 8, at 300 µM 12 ± 1%). Activation of the transmembrane dehydrogenase by menadione can also be demonstrated by measuring enzymatic reduction of ferricyanide to ferrocyanide in intact erythrocytes. Fig. 6 shows that menadione, at a concentration of 60 µM, rapidly increases the amount of ferrocyanide. After about 40 min, on the other hand,

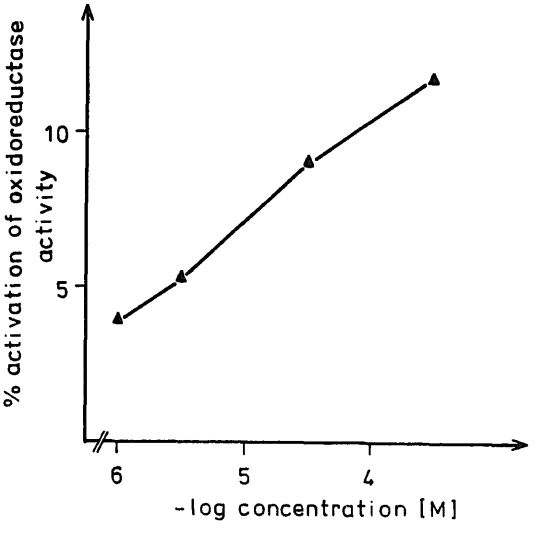

Fig. 8. Effect of menadione on oxidoreductase activity compared to control. The reduction of ferricyanide was measured in erythrocyte ghosts suspended in 150 mM $NaNO_3$, 1 mM KNO_3, 20 mM Hepes and 0.2 mM $K_3Fe(CN)_6$ at 37 °C. The reaction was started with 20 µM NADH. Data points are average values of four experiments (S.D. was within the size of the symbols).

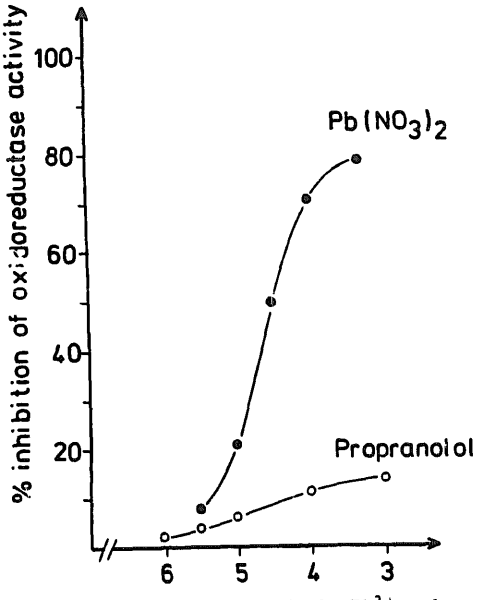

Fig. 9. Inhibition of oxidoreductase activity by Pb^{2+} and propranolol. Conditions are as described in Fig. 8, but with 0.2% Triton X-100.

the production of ferrocyanide levels off at values lower than the control values without menadione.

Effects of propranolol. In the preceding publication [3] on propranolol modulation of K^+ permeability, we demonstrated, in flux and patch-clamp experiments, that propranolol increases the sensitivity of the channel for Ca^{2+}. Fig. 6 demonstrates that, in contrast to the observations made with K^+ channels, the transmembrane dehydrogenase in intact cells is not affected by 1 mM propranolol. In addition to the transmembrane dehydrogenase activity in intact cells, we tested the effect of different concentrations of propranolol on the ferricyanide-NADH dehydrogenase activity in permeabilised ghosts. Again, there is much less effect of propranolol on oxidoreductase activity compared to the relatively high inhibitory effect of $Pb(NO_3)_2$ (see Fig. 9).

Discussion

Stimulation of K^+ permeability in human red cells by electron donors has been described previously [7–9]. In addition, involvement of a membrane-bound oxidoreductase in modulating K^+ permeability has been suggested [1]. The Ca^{2+}-activated K^+ permeability of human erythrocytes can be modulated by several substances that also affect membrane-bound oxidoreductase. This modulation of K^+ permeability has been demonstrated previously with probucol [10], vanadate and chloro-substituted naphthoquinone derivatives [1]. While vanadate stimulates the activity of both membrane systems, certain chloro-substituted naphthoquinone derivatives and probucol inhibit both systems. This contribution further demonstrates that oxidoreductase activity and K^+ permeability can be stimulated or inhibited in parallel if the K^+ channels are activated by Pb^{2+} instead of by Ca^{2+}. This concept is elucidated by the present experiments on the inhibitory effects of high Pb^{2+} concentrations and of Atebrin. Identical concentration dependencies could be detected, particularly for inhibition by Pb^{2+} (Fig. 5), supporting the view that modulation of the membrane-bound oxidoreductase regulates the Ca^{2+}- or Pb^{2+}-activated K^+ permeability [1].

As regards the effect of menadione on modulation of membrane-bound oxidoreductase and of Ca^{2+}- or Pb^{2+}-activated K^+ permeability, the situation is more complex. Fuhrmann et al. [1] showed that menadione stimulates Ca^{2+}- or Pb^{2+}-activated K^+ channels in freshly prepared cells and inhibits the Ca^{2+}-activated K^+ channels in ATP-depleted cells under Gardos-type conditions. Activation and inhibition of the Pb^{2+}-activated K^+ channels by menadione could also be demonstrated more directly by adding Pb^{2+} to a Ca^{2+}-free medium (Fig. 4). Depending on the time after initiation of metabolic depletion, activation or inhibition can be detected in intact cells. This observation suggests that the metabolic state of the cells determines whether or not menadione stimulates channel opening.

Instead of the expected stimulation of the membrane-bound oxidoreductase by menadione, as in microsomes [11], an inhibition of up to 27% was observed in erythrocyte ghosts permeabilized by Triton X-100. In this study, we demonstrate that, if Triton X-100 is omitted, menadione can activate the ferricyanide-NADH dehydrogenase in erythrocyte ghosts. The activation observed under these conditions is of the same order as that found by Talcott et al. [11] in microsomes. We conclude from this that activation by menadione was prevented, in earlier tests [1], by interference of Triton X-100. In close parallelism to the observations on K^+ permeability, either activation or inhibition by menadione occurred, again depending on the metabolic state of the cells. This is shown by the measurements of transmembrane dehydrogenase activity in intact cells (Fig. 6). Inhibition by menadione may be due to metabolic depletion of reduction equivalents of the cell. Since the Gardos effect has a latency of about 1 h, the transmembrane dehydrogenase is inactive. This results in an inhibitory effect of menadione on the relatively low K^+ efflux under these conditions [1]. Thus, the mode of action of menadione presents a further example for parallel modulation of K^+ permeability and oxidoreductase activity.

To cite yet another example from the recent literature on the parallel effects of drugs on membrane-bound oxidoreductase activity and Ca^{2+}-activated K^+ permeability in human red cells, in addition to our results with

Atebrin, the cholesterol-reducing drug probucol also inhibits both processes [10].

On to the substances mentioned above, the mechanism of propranolol, which can both activate and inhibit K^+ permeability, is distinct. Even at a propranolol concentration of 1 mM, there is only slight inhibition of about 10% in the dehydrogenase test with Triton X-100 permeabilised ghosts (Fig. 9), and no influence on the transmembrane dehydrogenase activity in intact cells (Fig. 6). Consequently, the membrane-bound oxidoreductase cannot be involved in the effects of propranolol on K^+ permeability.

The different effects of propranolol on the two membrane systems do not support the hypothesis that the membrane-bound oxidoreductase and the K^+ channel form a single biochemical membrane unit. We interpret these results as an indication that the activity of the oxidoreductase may influence the activation of the K^+-selective channels, but that the two membrane proteins are not identical.

The reverse statement that channel activity can affect dehydrogenase activity cannot be made. Low concentrations of propranolol stimulate K^+ permeability, but have no effect on the oxidoreductase. In addition, high concentrations of propranolol and K^+-free incubation of red cell ghosts lead to inhibition of the channel activity, but have only minor effects on the activity of the ferricyanide-NADH dehydrogenase.

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