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Effects of vanadate, menadione and menadione analogs on the Ca^{2+}-activated K^+ channels in human red cells. Possible relations to membrane-bound oxidoreductase activity

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The modulation of the Ca^{2+}- (or Pb^{2+}-)activated K^+ permeability in human erythrocytes by vanadate, menadione and chloro-substituted menadione analogs was investigated by measurements of K^+ fluxes and single-channel currents. Vanadate and menadione stimulate the K^+ permeability by increasing the probability of channel openings; the menadione analogs, on the other hand, inhibit the K^+ permeability by increasing the probability of channel closings. The compounds used in these experiments also interact with oxidoreductases; it is demonstrated that menadione analogs in contrast to menadione strongly inhibit the membrane-bound dehydrogenase in the erythrocytes. Concentrations of Pb^{2+} above 10 μmol/l, but not of Ca^{2+}, inhibit the enzyme activity as well as the K^+ permeability. The parallel effects on dehydrogenase activity and the K^+ channels suggest a direct relationship between these two systems in the membrane of erythrocytes.

Introduction

The cell membrane of human erythrocytes is relatively permeable to small anions, but relatively impermeable to small cations (for a review, see Ref. 1). Chloride and other anions are passively distributed across the cell membrane, whereas the distribution of Na^+, K^+ and Ca^{2+} is maintained by the balance of passive and active transport. Particularly for Ca^{2+}, a high gradient of more than four orders of magnitude is maintained; this is achieved by an extremely low permeability for Ca^{2+} of approx. $5 \cdot 10^{-5}$ mol/h per liter of cells [2] and by a powerful Ca^{2+}-extrusion pump in the cell membrane with a maximal transport capacity of $(5–10) \cdot 10^{-3}$ mol/h per liter of cells.

If this Ca^{2+} homeostasis is impaired so that the intracellular activity of Ca^{2+} raises to micromolar levels, a number of physical and biochemical mechanisms are triggered (see also Ref. 3). (1) Activation of a K^+-selective channel leading to a dramatic loss of K^+ into a medium containing low K^+ (for a review, see Refs. 4 and 5). (2) Inhibition of the membrane permeability for chloride and sulfate [6,7]. (3) Inhibition of the Na/K pump [8]. (4) Activation of the Ca pump [9]. (5) Activation of a cytoplasmic transglutaminase followed by crosslinking of γ-glutamyl ε-lysine residues of spectrin and other membrane proteins [10]. (6) Loss of cell deformability and irreversible changes of the shape of the erythrocyte [11,12]. (7) Breakdown of phospholipids in the cell membrane [13].

In this investigation we examined the activation of the Ca^{2+}-activated K^+ permeability of the

Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; Mops, 4-morpholinepropanesulphonic acid; CMNQ, chloromethyl-1,4-naphthoquinone.

membrane of human erythrocytes. In addition, we performed experiments with Pb^{2+} which easily enters the erythrocyte and like Ca^{2+} activates the K^+-selective channels [14]. However, high concentrations of Pb^{2+} in contrast to Ca^{2+} block the K^+ permeability. In human erythrocytes the number of the Ca^{2+}-activated channels has recently been determined to be in the range of 1 to 55 channels per cell [15]. This small number makes biochemical identification extremely difficult.

To characterize the channel further, we investigated the effects of different redox agents (vanadate, menadione and chloro-substituted menadione analogs) on ion fluxes as well as on single-channel currents. The menadione derivatives have been synthesized and investigated especially with respect to their antitumor properties by Sartorelli and co-workers [16–19]. Recently, it was demonstrated that these and similar sulfhydryl-reactive derivatives of menadione inactivate microsomal NADPH-cytochrome *c* reductase [20]. Since the above-mentioned agents are known to interfere with redox mechanisms, we also investigated their influence on membrane-bound oxidoreductase activity as revealed by ferricyanide reduction in open membrane preparations of human erythrocytes. From the parallel effects on the Ca^{2+}-activated K^+ channel and the oxidoreductase a possible relationship is deduced.

Methods

Cell preparation and flux experiments

Human erythrocytes with heparin as anticoagulant were washed three times in 150 mmol/l NaCl, 1 mmol/l KCl or in 150 mmol/l $NaNO_3$, 1 mmol/l KNO_3; the pH of the solutions was adjusted to 7.6 by 20 mmol/l Hepes. The cells were suspended to 0.5% in this solution at 37°C. The Ca^{2+}-activated K^+ permeability was stimulated by Pb^{2+} or by elevation of the activity of intracellular Ca^{2+}. The latter was achieved either by metabolic depletion and hence by inhibition of the ATP-driven Ca^{2+}-pump as originally described by Gardos [21], or by addition of the Ca^{2+} ionophore A23187 to the medium. For the activation of the K^+-selective channels by Pb^{2+} neither metabolic depletion nor addition of A23187 is necessary, since lead easily permeates the cell membrane probably as a complex in its lipid-soluble undissociated form. All three procedures were used to study the effects of menadione and its derivatives on the K^+ permeability and in case of the experiments with metabolically depleted cells also of vanadate. At various times after the K^+ permeability was elicited samples of the cell suspension were added to an ice-cold solution of 113 mmol/l $MgCl_2$ [22], and the cells were washed three times with this solution. The cell contents of Na^+ and K^+ were determined by flame photometry and expressed per kg hemoglobin; the hemoglobin was measured at the isosbestic point for oxy- and methemoglobin at 527 nm [23]. In order to avoid limitation of the stimulated high K^+ fluxes by less permeable anions, we substituted chloride by the highly permeable anion nitrate [22]. To demonstrate that the effects were specific on the K^+-selective channels, changes of the Na^+ permeability were also examined.

Measurements of ferricyanide-NADH dehydrogenase activity

The enzyme activity was determined as reduction of ferricyanide per mg protein in erythrocyte ghosts permeabilized by Triton X-100 and suspended at 37°C in nitrate medium as used for the flux measurements. Ghosts were prepared according to the method of Dodge et al. [24], and the content of protein was determined by the procedure of Lowry et al. [25] with bovine serum as standard. Ferricyanide-NADH dehydrogenase was measured in an Aminco DW_2 double-beam spectrophotometer (American Instrument Co., Silverspring, MD, U.S.A.) using the dual-wavelength mode to subtract absorbance at 485 nm from 420 nm [26].

Measurements of single-channel currents

Single-channel currents of the Ca^{2+}-activated K^+ channels were recorded from cell-free inside-out membrane patches [27] by the improved Gigaseal patch-clamp technique [28]. The pipette solution in contact with the external membrane surface and the control bath solution in contact with the internal membrane surface contained 150 mmol/l KCl, 1 mmol/l $MgCl_2$, 10 μmol/l $CaCl_2$, and were adjusted to pH 7.4 by 10 mmol/l Mops. Drugs were added to give the indicated concentra-

tions. All experiments were performed at 22°C.

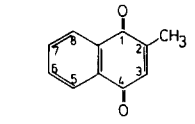
The currents were recorded at a constant holding potential of usually −100 mV and stored on analog magnetic tape. The unphysiological holding potential and high external K^+ concentration were used to increase single-channel currents to levels that can easily be analysed (see Ref. 29). For analysis, the data were transferred to a brush recorder (Gould Electronics, Cleveland, OH, U.S.A.) and evaluated by hand, or the records were digitized at a sampling rate of 2 kHz (Data Translation, Natick, MA, U.S.A.), stored on floppy diskette and evaluated by means of an LSI 11/23 computer (Digital equipment, Maynard, MA, USA). For dwell-time distributions records with only one active channel were analysed, and it is assumed that the channel opens or closes if the recorded current passes the value of half the amplitude of a single-channel event.

Materials

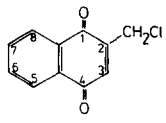
$NaVO_3$ p.a., Triton^R X-100, $NaNO_3$ p.a., $Pb(NO_3)_2$ p.a., and menadione were obtained from Merck (Darmstadt, F.R.G.), NADH p.a., Hepes p.a. from Serva (Heidelberg, F.R.G.), A23187 in acid from Calbiochem-Behring (Frankfurt am Main, F.R.G.). The menadione analog chloro-substituted compounds were a gift from Dr. A.C. Sartorelli, Department of Pharmacology, Yale University (New Haven, CT, USA). All other reagents were of analytical grade. Menadione (2-methyl-1,4-naphthoquinone) and its derivatives (see Table I) were solubilized in ethanol; in all solutions the final concentration of ethanol was 0.7%. If not explicitely stated, the results are not affected by the presence of ethanol as determined in experiments with control solutions without and with ethanol.

TABLE I

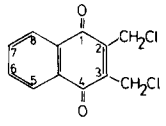
STRUCTURE OF MENADIONE AND MENADIONE ANALOG CHLORO-SUBSTITUTED SUBSTANCES



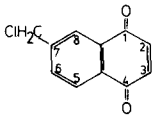
2-Methyl-1,4-naphthoquinone
MENADIONE



2-Chloromethyl-1,4-naphthoquinone
2-CMNQ



2,3-Bis(chloromethyl)-1,4-naphthoquinone
2,3-CMNQ



7-Chloromethyl-1,4-naphthoquinone
7-CMNQ

Results

Before the effects of menadione and the menadione analogs on the Ca^{2+}-activated K^+ permeability were analysed, possible modulations of the ferricyanide-NADH dehydrogenase activity in the erythrocytes were tested.

Inhibition of ferricyanide-NADH dehydrogenase activity by menadione and chloro-substituted menadione analogs

Fig. 1 depicts the effects of menadione, 2-

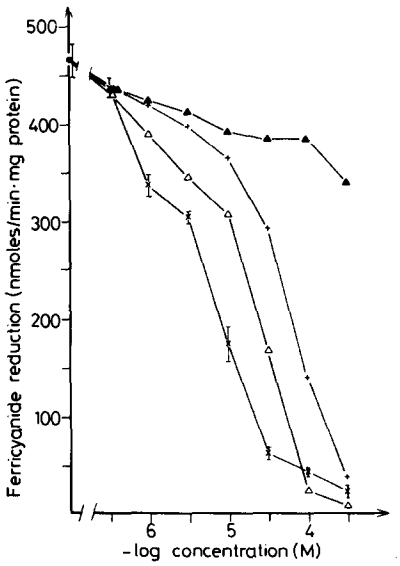


Fig. 1. Effects of menadione and menadione analogs on ferricyanide reduction in erythrocyte ghosts. 22.5 μg/ml membrane protein was suspended in nitrate solution with 0.2 mmol/l potassium ferricyanide and 0.2% Triton X-100. The reaction was started with 20 μmol/l NADH. ▲——▲, menadione; +——+, 2-CMNQ; ×——×, 2,3-CMNQ; △——△, 7-CMNQ. The points represent the mean of two experiments and the points with ±S.D.-bars the mean of four experiments.

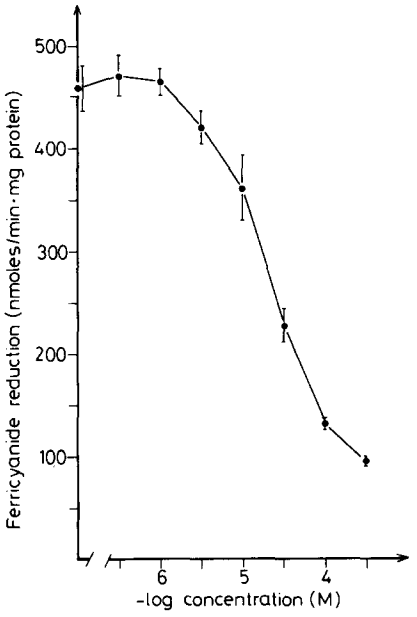


Fig. 2. Effect of $Pb(NO_3)_2$ on ferricyanide reduction in erythrocyte ghosts. Assay as for Fig. 1. Points represent mean values of four experiments ± S.D.

CMNQ, 2,3-CMNQ and 7-CMNQ on ferricyanide-NADH dehydrogenase activity in the membranes of human erythrocytes. The strongest inhibition is produced by 2,3-CMNQ with an IC_{50} of 5 μmol/l followed by 7-CMNQ ($IC_{50} = 18.6$ μmol/l) and 2-CMNQ ($IC_{50} = 50.1$ μmol/l). For all three compounds inhibition is already visible at 1 μmol/l and is nearly complete at the highest investigated concentrations of 300 μmol/l. Menadione only slightly reduces the enzyme activity, and even at 300 μmol/l the inhibition is only 27%.

Effects of Pb^{2+} and Ca^{2+} on ferricyanide-NADH dehydrogenase activity

At concentrations in the micromolar range Pb^{2+} activates the same K^+ channels in human erythrocytes that are activated by Ca^{2+} [14,30]. But in contrast to Ca^{2+}, high concentrations of Pb^{2+} inhibit the K^+ permeability [14]. Therefore, we analysed the effects of these two cations on the ferricyanide-NADH dehydrogenase activity under the same conditions as described above. Fig. 2 demonstrates that Pb^{2+} also has an inhibitory effect on the enzyme activity with an IC_{50} of 30

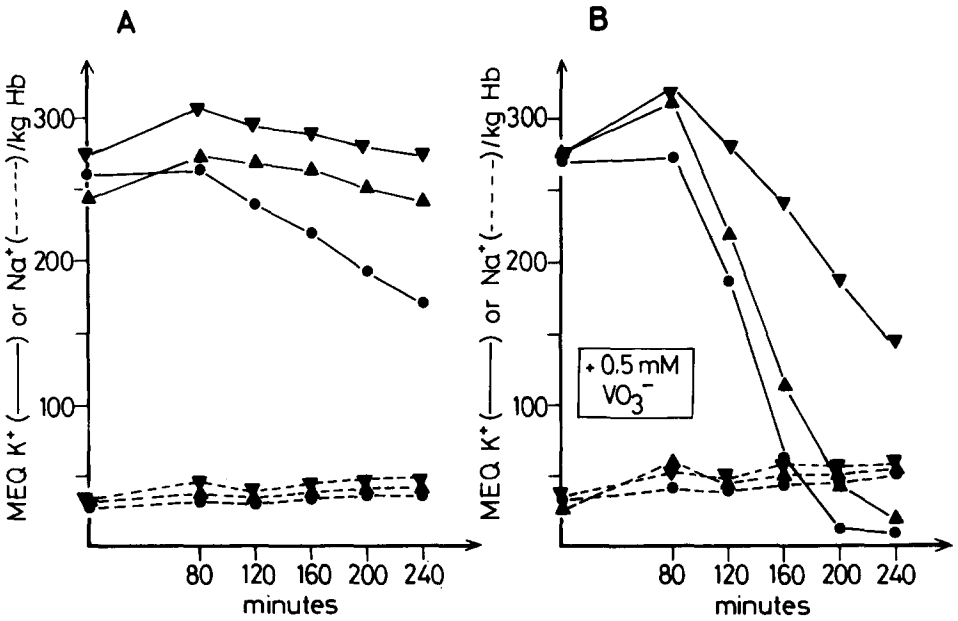


Fig. 3. Effect of menadione on K^+ (————) and Na^+ (— — —) content of erythrocytes as a function of time. Erythrocytes (0.5% hematocrit) were suspended in nitrate solution with 0.5 mmol/l $CaCl_2$, 2 mmol/l adenosine, 2.5 mmol/l iodoacetate. Experiments were started by adding the cells. Fluxes were measured in the absence (Fig. 3A) or presence of 0.5 mmol/l VO_3^- (Fig. 3B). ●————●, control without menadione; ▲————▲, 3 μmol/l menadione; ▼————▼, 30 μmol/l menadione.

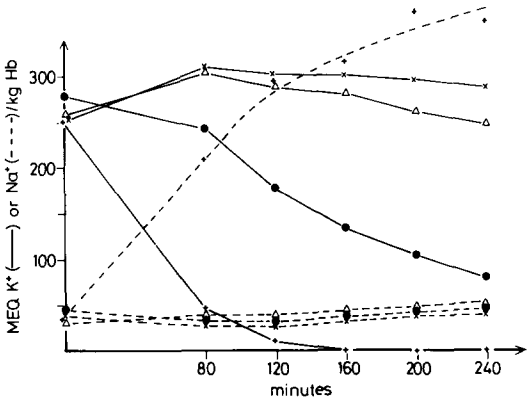


Fig. 4. Effect of menadione analogs on K^+ (————) and Na^+ (— — —) content of erythrocytes as a function of time. Assay as for Fig. 3. ●————●, control without menadione analog; +————+, 25 μmol/l 2-CMNQ; ×————×, 25 μmol/l 2,3-CMNQ; △————△, 25 μmol/l 7-CMNQ.

μmol/l. Ca^{2+} concentration up to 600 μmol/l, on the other hand, do not influence the dehydrogenase activity.

Effects of menadione and chloro-substituted menadione analogs on cation fluxes in ATP-depleted erythrocytes

The Ca^{2+}-activated K^+ efflux was induced under conditions (see legend to Fig. 3) similar to those described by Gardos [21]. Fig. 3A demonstrates that the Ca^{2+}-activated K^+ permeability is inhibited by 3–30 μmol/l menadione. As already shown previously [22,31], addition of 0.5 mmol/l VO_3^- further stimulates the K^+ permeability in the ATP-depleted cells (compare also Fig. 3A with Fig. 3B). Menadione inhibits the K^+ efflux also under these conditions (Fig. 3B).

Inhibition of the K^+ permeability in the metabolically depleted erythrocytes was also found with the menadione derivatives 7-CMNQ and 2,3-CMNQ (see Fig. 4). Already at 25 μmol/l a nearly complete inhibition was obtained. The compound 2-CMNQ, on the other hand, produces an increase of permeability; this, however, is not selective for K^+, and the membrane also becomes permeable for Na^+. In the presence of 0.5 mmol/l VO_3^- similar effects were seen with the three menadione derivatives (not shown).

Effects of menadione and chloro-substituted menadione analogs on cation fluxes after addition of the ionophore A23187

Ca^{2+} contamination in the flux medium amounts to a few μmol/l, and is sufficient to stimulate the Ca^{2+}-activated K^+ permeability in

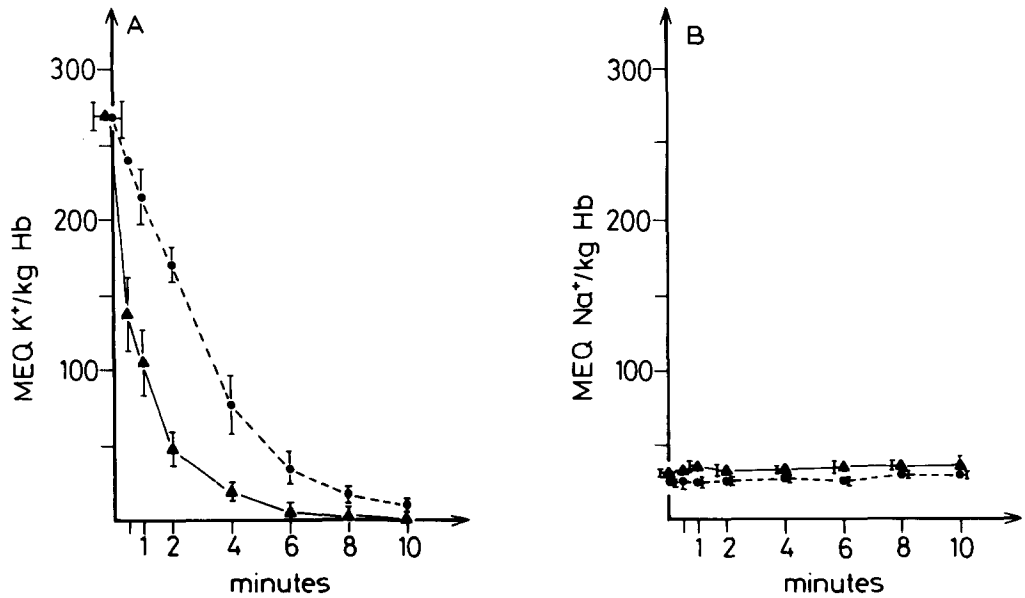


Fig. 5. Effect of menadione on K^+ and Na^+ content of erythrocytes as a function of time. The experiments were started by addition of 0.5 μmol/l A23187. ●— — —●, control without menadione; △————△, 300 μmol/l menadione. Points represent mean values of four experiments ± S.D.

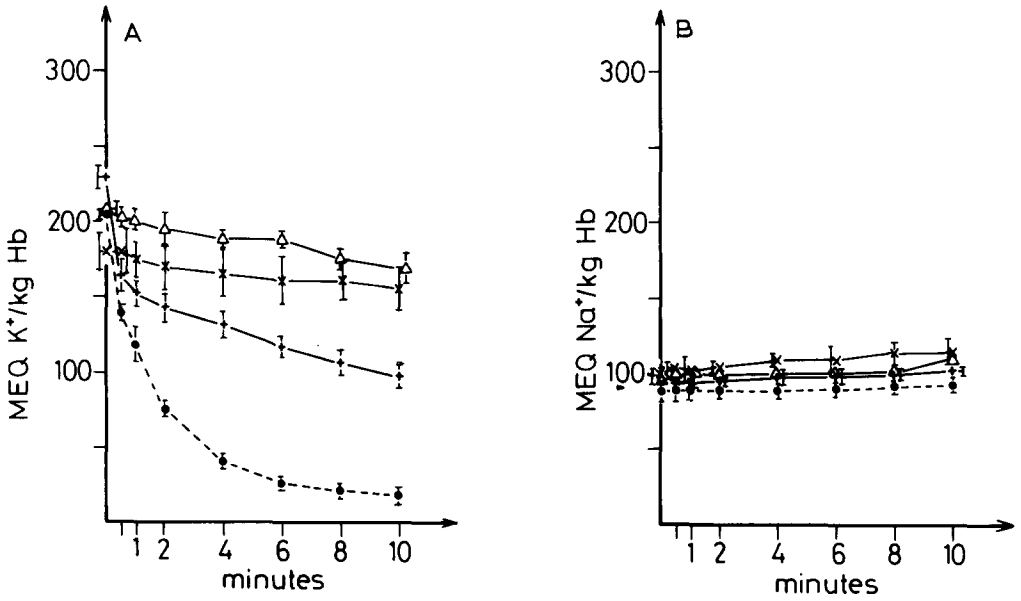


Fig. 6. Effect of menadione analogs on K^+ and Na^+ content of erythrocytes as a function of time. The experiments were started by addition of 1 μmol/l A23187. ●— · · —● control without menadione analog; △———△, 300 μmol/l 7-CMNQ; +———+, 150 μmol/l 2-CMNQ; ×———×, 150 μmol/l 2,3-CMNQ. Points represent mean values of four experiments ± S.D. In this experiment cells have been stored overnight in saline solution at 4°C, thus the K^+ content is lower and the Na^+ content is higher than normal.

freshly prepared erythrocytes after addition of the Ca^{2+} ionophore A23187 (Fig. 5). If 300 μmol/l menadione were added before application of A23187, the K^+-selective permeability was even further stimulated.

Application of the three menadione derivatives in the presence of A23187 shows specific inhibition of the K^+ permeability (Fig. 6) though the concentrations used are by nearly an order of magnitude higher than those used in the experiments with ATP-depleted eryrthrocytes. For 2,3-CMNQ the inhibition of K^+ efflux shows nearly the same dependence on concentration as the inhibition of ferricyanide oxidoreductase activity (Fig. 7).

Effects of menadione and chloro-substituted menadione analogs on cation fluxes after application of $Pb(NO_3)_2$

Enhancement of the K^+ fluxes by menadione is also seen if in freshly prepared erythrocytes the K^+ permeability is activated by addition of 20 μmol/l $Pb(NO_3)_2$. In these experiments the ethanol already increased the K^+ efflux. However,

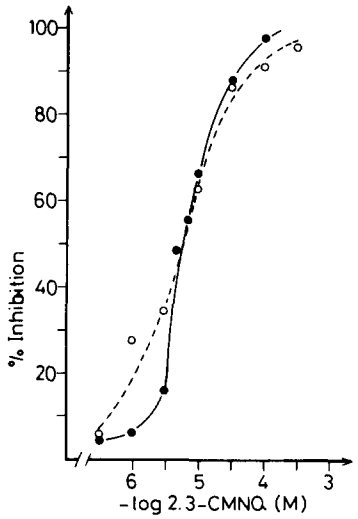


Fig. 7. Comparison of the inhibitory effects of 2,3-CMNQ on ferricyanide oxidoreductase activity (○— — —○) (experimental data from Fig. 1, 22.5 μg/ml membrane protein) and on K^+ efflux (●———●) induced by 1 μmol/l A23187 in NO_3^- flux medium. The K^+ efflux was monitored by right-angular light scattering (method given in Refs. 14, and 22, 5 μg/ml membrane protein). Points represent mean values of four experiments; the S.D. values are within the size of the symbols.

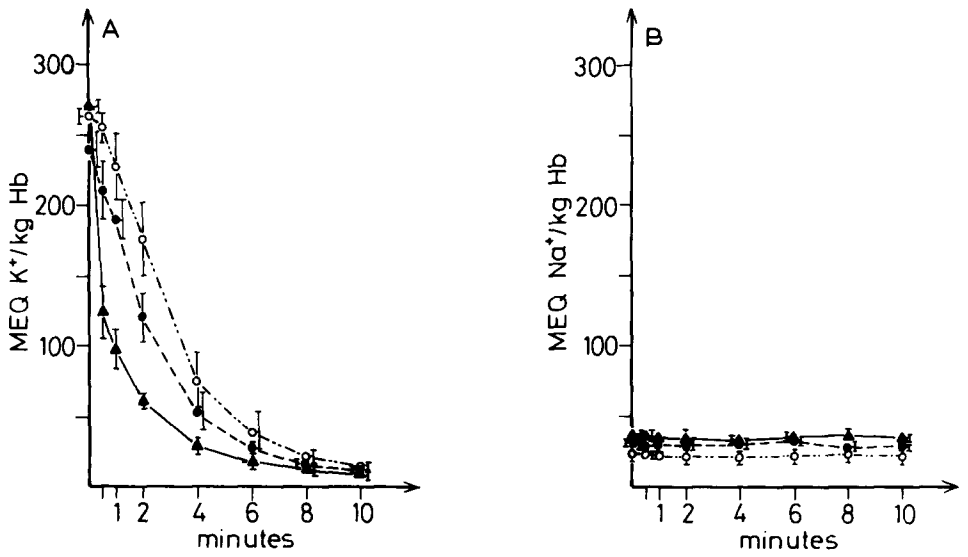


Fig. 8. Effect of menadione on K^+ and Na^+ content of erythrocytes as a function of time. The experiments were started by addition of 20 μmol/l $Pb(NO_3)_2$. O·–·–·O, control; ●— — —●, control + 100 μl ethanol; ▲———▲, 300 μmol/l menadione. Points represent mean values of four experiments ± S.D.

application of menadione further stimulates the K^+ permeability (Fig. 8). A slightly different effect is observed with the menadione derivatives when the K^+ permeability is activated by Pb^{2+} compared to ATP-depleted cells or to activation by Ca^{2+} and the ionophore A23187. While the two compounds 7-CMNQ and 2,3-CMNQ also inhibit the K^+ efflux, 2-CMNQ initially increases the K^+ permeability as seen in ATP-depleted erythrocytes (Fig. 9), but about 4 min after application of the drug an inhibitory effect occurs as seen in the experiments with Ca^{2+} and the ionophore.

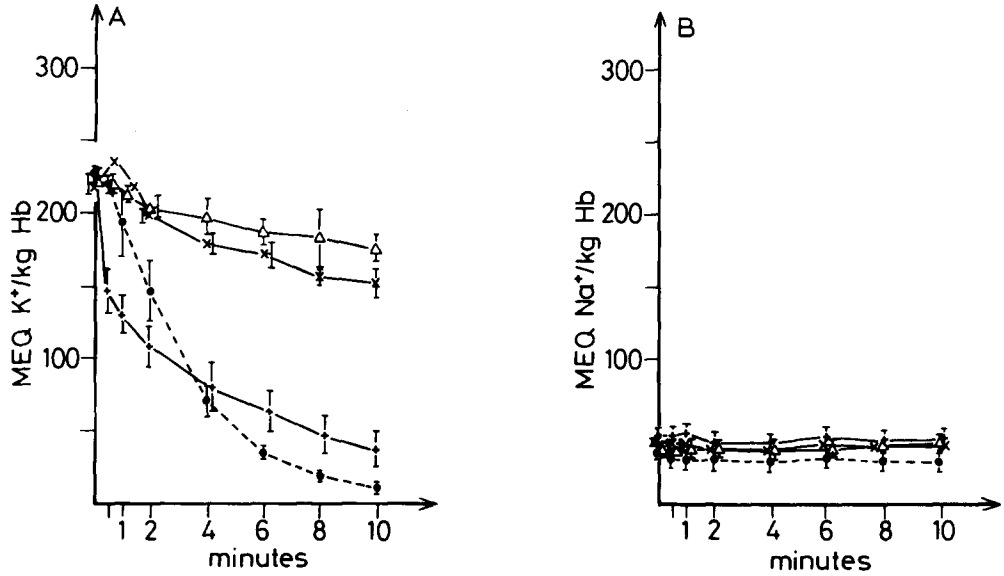


Fig. 9. Effect of menadione analogs on K^+ and Na^+ content of erythrocytes as a function of time. The experiments were started by addition of 20 μmol $Pb(NO_3)_2$. ●— — —●, control without menadione analog; △———△, 150 μmol/l 7-CMNQ; + ———— +, 150 μmol/l 2-CMNQ; × ———— ×, 150 μmol/l 2,3-CMNQ. Points represent mean values of four experiments ± S.D.

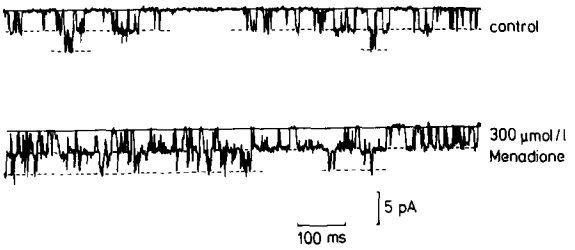


Fig. 10. Effect of 300 µmol/l menadione on the activity of single-channel events measured at a holding potential of −100 mV. The solid horizontal lines indicate the closed state, dotted lines indicate the states of one or two open channels. Exp. RC233.

Effect of vanadate, menadione and chloro-substituted menadione analogs on single-channel K^+ currents

Fig. 10 shows inward currents through single K^+-selective channels under control conditions and in the presence of 300 µmol/l menadione in the bath solution. Menadione does not affect the am-

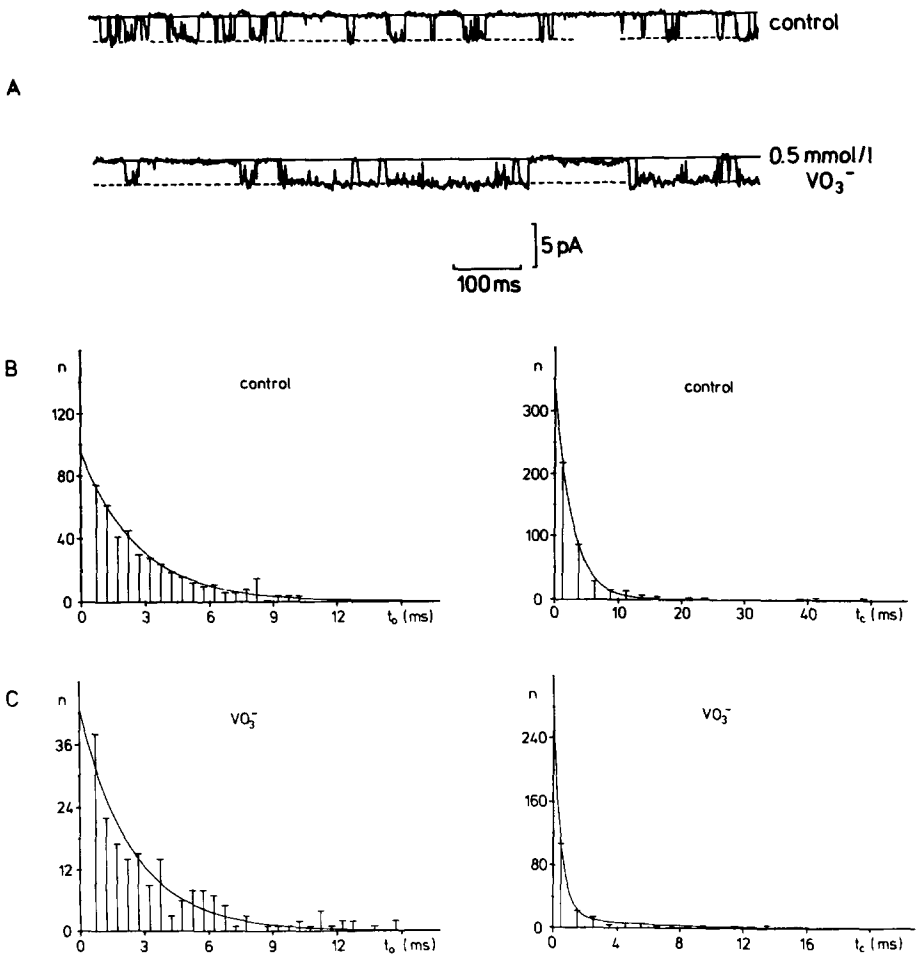


Fig. 11. Effect of VO_3^- on single-channel events measured at a holding potential of −100 mV. Exp. RC 242. Single-channel records Fig. 11A; distribution of open times and of closed times in control solution Fig. 11B and with 0.5 mmol/l VO_3^- Fig. 11C. Solid curves are least-squares fits of $P_0 e^{-t/\tau_o}$ for the open times and $P_1 e^{-t/\tau_{c1}} + P_2 e^{-t/\tau_{c2}}$ for the closed times. The parameters are:

	P_0	τ_o (ms)	P_1	τ_{c1} (ms)	P_2	τ_{c2} (ms)
Control	94	5.3	346	5.4		
Vanadate	43	5.0	261	1.0	15	9.5

TABLE II

VALUES FOR THE PROBABILITY OF A CHANNEL TO BE OPEN

Determined at a holding potential of −100 mV.

	p (control)	p (drug)	Δp (%)
300 μmol menadione per l	0.38	0.42	+10.5
500 μmol vanadate per l	0.34	0.42	+23.5
20 μmol 7-CMNQ per l	0.23	0.20	−13.0

plitude of the single-channel currents, but the probability of a channel to be open, p, is increased. A similar result was obtained after application of 0.5 mmol/l VO_3^- to the bath medium (Fig. 11A). p-values as calculated from the mean values of open and closed times according to the formula

$$p = \tau_o/(\tau_o + \tau_c)$$

are listed in Table II. More detailed analysis of the

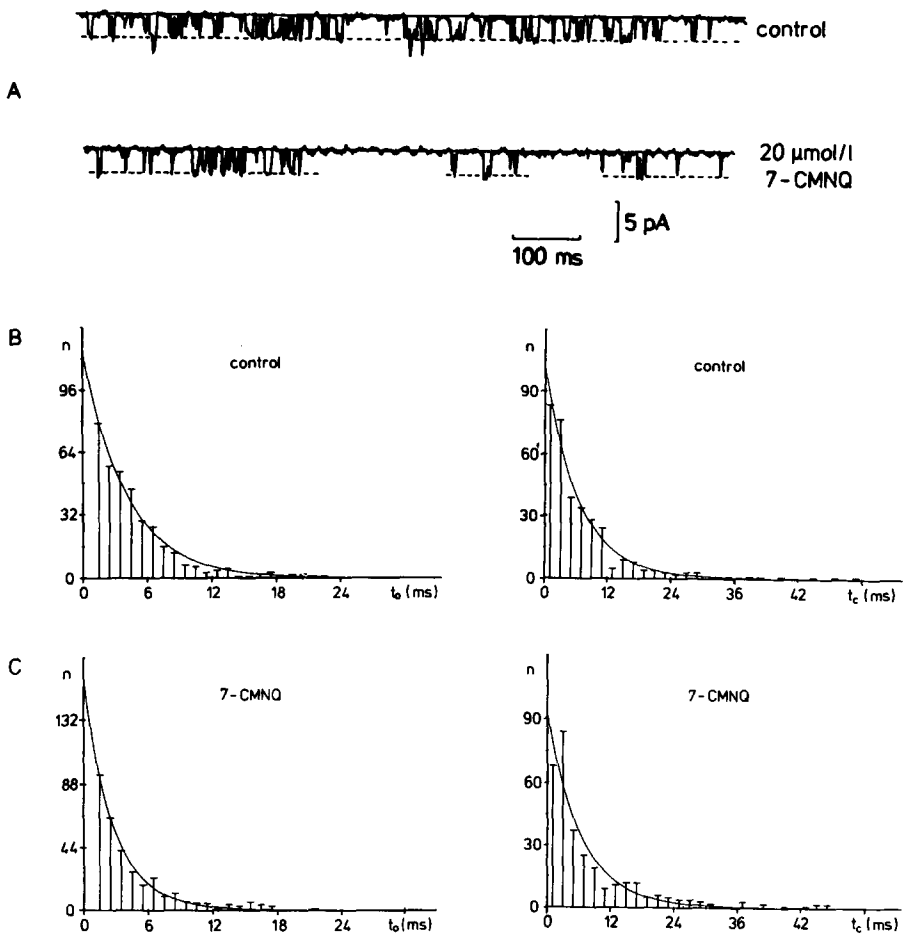


Fig. 12.Effect of menadione analog 7-CMNQ on single-channel events measured at a holding potential of −100 mV. Exp. RC240. Single-channel records Fig. 12A; distribution of open times of closed times in control solution Fig. 12B and with 20 μmol/l 7-CMNQ Fig. 12C. Solid curves are least-squares fits (see legend to Fig. 11). The fitted parameters are:

	P_0	τ_o (ms)	P_1	τ_{c1} (ms)
Control	116	4.0	102	6.5
7-CMNQ	160	2.8	92	6.7

open and closed times reveals that the increased probability of the open state is primarily due to an increased probability of channel openings. Figs. 11B and C show dwell-time histograms of the open and closed state of a single channel without and with VO_3^- in the solution in contact with the internal membrane surface. While the open-time distribution is nearly unaffected and can be described by time constants of 5.3 and 5.0 ms, respectively, the mean dwell-time of the closed state decreases from 9.9 ms to 7.0 ms after application of VO_3^- (see also legend to Fig. 11). Qualitatively, the same observations have been made with menadione.

Similar to the flux measurements, the menadione derivatives 7-CMNQ and 2,3-CMNQ show an inhibitory effect on the K^+ currents. Fig. 12A gives an example for 7-CMNQ that reduces the probability of a channel to be open (see also legend to Fig. 12). With these inhibitors the probability of channel closings increases. This becomes most obvious at a holding potential of -150 mV, where the *p*-value decreases from 0.38 to 0.29 (compare also Table II for the holding potential at -100 mV). Fig. 12B and C show dwell-time distributions and demonstrate that the closed-times are nearly unaffected by 7-CMNQ, while the open-time distributions can be described by a time constant of 4.0 and 2.8 ms without and with 20 μmol/l 7-CMNQ.

Discussion

Modulation of the K^+ permeability in human erythrocytes by the cytoplasmic NADH/NAD ratio [32] and stimulation by electron donors has been investigated previously [33,34]. However, an involvement of the NADH dehydrogenase of the plasma membrane in Ca^{2+}-activated K^+ transport has not been found [35]. Our results on the other hand, demonstrate that the tested substances that modulate the Ca^{2+}-activated K^+ channel in the membranes of human erythrocytes also influence the membrane-bound oxidoreductase.

In this investigation we demonstrate that the menadione chloro-substituted substances 2-CMNQ, 2,3-CMNQ, and 7-CMNQ strongly inhibit the dehydrogenase activity in the erythrocytes. In the flux experiments the menadione chloro-substituted compounds 2,3-CMNQ and 7-CMNQ produce a strong specific inhibition of the Ca^{2+}-activated K^+ channels. For 2,3-CMNQ the same dependence on the concentration of the inhibitor was demonstrated for the K^+ channel and the dehydrogenase activity (Fig. 7). 20 μmol/l 7-CMNQ also produce partial inhibition (10–20%, see Table II) of the single-channel activity, and at a concentration of 210 μmol/l (comparable to the concentrations used in the flux experiments) single-channel openings became such a rare event that they have not been analysed. Thus, there is nearly quantitative agreement between the flux and patch-clamp data from experiments with 7-CMNQ. For the other compounds only qualitative agreement could be demonstrated. This can be explained by the different experimental conditions like temperature (37°C and 22°C), different solutions and finally differences in the membrane preparation (intact erythrocytes with redox systems like NADH or gluthadion and open membrane patches). The analysis of single-channel events at the lower concentration of the inhibitor demonstrates that the closed times are nearly unaffected by 7-CMNQ, but the open times are reduced. Thus, the inhibitory effect seen in the flux experiments can be explained by a direct action of the menadione derivatives on the K^+ channel by increasing the probability of channel closings.

In addition to the above-mentioned substances, we tested the effect of $Pb(NO_3)_2$ on the ferricyanide-NADH dehydrogenase activity of erythrocyte membranes. The inhibitory effect of Pb^{2+} on ferricyanide-NADH dehydrogenase activity parallels the inhibition of the K^+ permeability [14] by high concentrations of Pb^{2+}. On the other hand, high concentrations of Ca^{2+} neither inhibit the enzyme activity nor the K^+ fluxes or currents. Pb^{2+} concentrations of a few μmol/l have nearly no effect on the enzyme activity, and may even stimulate the K^+ permeability.

It is well known that Pb^{2+} not only binds to PO_4^- and COOH-containing ligands, but also to SH-groups [36]. The presence of functional SH-groups of the ferricyanide-NADH dehydrogenase may be assumed since *p*-chloromercuryphenylsulfonate at a concentration of 25 μmol/l inhibits the enzyme by about 90% (results not shown). Also the inhibition by the sulfhydryl-reactive

derivatives of menadione would be in favour of such functional SH-groups. For microsomes it was postulated [20] that the loss of reductase activity is related to a displacement of electronegative groups attached to the allylic carbon of the naphthoquinone derivatives by microsomal nucleophiles. Sulfhydryl groups have been assumed to be involved in the inactivation of the reductase. The IC_{50} values are similar to those observed for the NADPH-cytochrome reductase in microsomes of mouse liver [20]. A similar mechanism might also be responsible for the modulation of the dehydrogenase activity in the membrane of erythrocytes.

Application of 2-CMNQ obviously results in more unspecific effects in the flux experiments with energy-depleted cells (see Fig. 4) by increasing the permeability of both K^+ and Na^+. In this respect 2-CMNQ is similar to *p*-chloromercuryphenylsulfonate and other mercury compounds [37]. Therefore, 2-CMNQ is of minor importance in this investigation.

Menadione, in contrast to the menadione analogs 7-CMNQ and 2,3-CMNQ, stimulates the K^+ permeability if the channels are activated by Ca^{2+} or Pb^{2+} in freshly prepared cells, or if single-channel currents are analysed. This stimulation is compatible with the oxidation of menadione by the NADPH-cytochrome reductase in microsomes with ferricyanide [20]. Also in the dehydrogenase test menadione does not produce the strong inhibition as observed with the chloro-substituted compounds; only a slight inhibition of not more than 27% can be observed. We found an exception in the experiments with ATP-depleted cells, where menadione produced strong inhibition of the K^+ flux. We have no definite explanation for this inhibitory effect in the metabolically depleted cells, but the different metabolic states of the cells may influence the activity or sensitivity of the K^+ channels. The fact that the probability of a single channel to be open is increased by menadione demonstrates a direct effect of this compound on the K^+ channels. The quantitative difference of the stimulating effect of menadione in the flux and the patch-clamp experiments may be due to differences in the intracellular Ca^{2+} activity; the patch-clamp experiments were performed at 10 μmol/l Ca^{2+}; at lower activity (2 μmol/l) the stimulation by menadione was much higher (results not shown).

Parallelism between the effects on dehydrogenase activity and Ca^{2+}-activated K^+ permeability may also be deduced from the effects of VO_3^-. It has been demonstrated in membranes of erythrocytes and microsomes that VO_3^- markedly stimulates enzymatic NADH oxidation [38,39] similar to menadione that is oxidized by the NADPH-cytochrome P-450 reductase in microsomes [40].

In two recent publications [22,31] it was shown that VO_3^- strongly and selectively stimulates the K^+ permeability in ATP-depleted erythrocytes; this phenomenon is also depicted in Fig. 3. It could be excluded [22] that the reduced form of VO_3^-, the vanadyl cation, is responsible for this effect, and the possibility was discussed that VO_3^- indirectly affects the Ca^{2+}-activated K^+ permeability by an inhibition of the Ca^{2+} pump that would increase the intracellular Ca^{2+} activity. In this investigation we cannot exclude this as an additional possibility, but we definitely demonstrated by the patch-clamp experiments that VO_3^- directly acts on the Ca^{2+}-activated K^+ channel in a manner similar to menadione; the open time distribution is nearly unaffected, but the closed times are reduced leading to an increase of the probability of the open state by about 25%. This increase is lower than expected from the flux experiments (see Fig. 3), but can be explained by the different experimental conditions as mentioned above.

In summary, we have shown that menadione and VO_3^- act on the K^+ channel by increasing the probability of channel openings and that 2,3-CMNQ and 7-CMNQ increase the probability of channel closings. These direct actions on the single-channel activity can account for the effects seen in the flux experiments. However, an exception is the inhibitory effect of menadione on K^+ fluxes in the ATP-depleted erythrocytes. The inhibition of the K^+ channels by high concentrations of Pb^{2+} and of the ferricyanide-NADH dehydrogenase, as well as the inhibition of K^+ channels and the dehydrogenase activity by 2,3-CMNQ and 7-CMNQ suggest a possible relationship between the Ca^{2+}-activated K^+ channel and the membrane-bound dehydrogenases. However, further experiments are needed for more definitive conclusions; such experiments are currently in progress in our laboratory.

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