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The effect of ferricyanide with iodoacetate in calcium-free solution on passive cation permeability in human red blood cells: comparison with the Gardos-effect and with the influence of PCMBS on passive cation permeability

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Freshly prepared human red blood cells incubated with 5 mM ferricyanide, 0.2 mM iodoacetate and 2 mM adenosine in the presence of 5 mM EGTA demonstrate comparable increases in Na^+ and K^+ permeability (ferricyanide effect). This effect is unrelated to the Ca^{2+}-activated K^+ channel (Gardos effect) since influx of Ca^{2+} from outside the cell is excluded. Also this effect is different from the non-specific Na^+ and K^+ permeability change elicited by PCMBS. These differences become obvious by using various reagents. For example, A23187 and quinidine exert opposite effects in Gardos and ferricyanide experiments, where A23187 and atebrin react oppositely in the latter and in PCMBS experiments. The ferricyanide effect described here does not involve formation of nonspecific channels. The change in Na^+ permeability separately from K^+ permeability under certain circumstances suggests a more specific effect.

Introduction

In red blood cells depleted of ATP with iodoacetate (IAA) and adenosine, calcium can induce potassium permeability (Gardos effect) while sodium permeability remains nearly unchanged (for reviews, see Ref. 1–3). In contrast to this selective potassium permeability change, substances like mercury and organic mercurials react with sulfhydryl groups of red cell membranes to produce a loss of potassium, a gain of sodium and finally hemolysis [4].

In 1963 Passow and Gruner [5,6] studied the combined action of a small amount of IAA (0.12 mM) and ferricyanide (5 mM) on calcium-dependent potassium permeability in human red cells. If these substances were applied with 10 mM adenosine and 0.5 mM calcium, a change in potassium permeability of the cells was found to occur comparable to that in the Gardos-type experiments.

Since ferricyanide is not able to penetrate the red cell [7] and glycolysis was only partially blocked by the small concentration of IAA, the conclusion was drawn that two sets of membrane SH-groups, one for ferricyanide and another for IAA, are involved in the potassium permeability, with the one for ferricyanide being located at the external cell surface. In addition to the permeability change, Mishra and Passow [7] demonstrated in the presence of 0.12 mM IAA and 5 mM adenosine a reduction of ferricyanide to ferrocyanide via a membrane bound NADH dehydrogenase together with intracellular ATP synthesis.

In this investigation we studied potassium permeability in human red cells under experimental conditions similar to those described by Passow and Gruner, but in addition we also measured sodium permeability. We found that the permeability change was not selective for potassium, since there is also an increase in sodium permeability. The change in sodium permeability was not dependent on the presence of calcium and the change in potassium permeability was only slightly stimulated by calcium.

Abbreviations: EGTA, ethyleneglycol bis-(2-aminoethyl ether)-*N,N'*-tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazinecthanesulfonic acid; IAA, iodoacetate; mequiv., milliequivalents; PCMBS, *p*-chloromercuribenzene sulfonate.

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The permeability change of potassium and sodium in calcium-free medium induced by ferricyanide in the presence of IAA and adenosine will be called 'ferricyanide effect'. In brief reports [8,9] we previously demonstrated the ferricyanide effect in human red cells. The aim of this study is to investigate the similarities and differences between the 'ferricyanide effect', the Gardos effect and the influence of organic mercurials like PCMBS on potassium and sodium permeability in human red blood cells.

Materials and Methods

Materials

The Ca^{2+}-ionophore A23187 in acid form was purchased from Calbiochem-Behring (Frankfurt am Main, F.R.G.). Quinidine and adenosine were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Hepes and iodoacetate as a sodium salt were purchased from Serva (Heidelberg, F.R.G.), EGTA and inosine from Fluka (Buchs, Switzerland). All other chemicals were of analytical grade.

Methods

Human red blood cells freshly drawn with heparin as anticoagulant were washed three times in ice cold 150 mM NaCl, 1 mM EDTA and 20 mM Hepes at pH 7.4 and made up to a hematocrit of 50%. For experiments with calcium we omitted the 1 mM EDTA in the washing solution. Net flux measurements of potassium and sodium were done with 0.5% cells incubated at 37 °C in the medium given in the experiments. Most experiments were run in a Hepes buffer at pH 7.4 (37 °C) containing 5 mM EGTA in calcium-free experiments. At various times samples of the cell suspension were added to ice cold 113 mM $MgCl_2$ solution and the cells were washed three times with this solution in order to remove extracellular potassium and sodium. After hemolysing the cells by addition of 0.01% lithium and by ultrasound, intracellular sodium and potassium were determined by flame photometry and hemoglobin was measured at its isosbestic point at 527 nm [10].

Results

Effects on potassium and sodium permeability

The first experiment is similar to that of Gruner and Passow [5,6] in which the combined action of 5 mM $K_3Fe^{III}(CN)_6$ and 0.2 mM IAA in the presence of 2 mM adenosine and 0.5 mM calcium on potassium and sodium permeability is investigated (Fig. 1). After a lag period of 2 h there is a potassium efflux comparable to that measured by Passow [6]: 97 mequiv./kg hemoglobin per h (between 120 and 200 min) in our experiment, which is 32.4 mmol K^+/l cells per h compared to 36 mmol K^+/l cells per h in Passow's experiment. The sodium influx also increases after the same lag period of 2 h: 41 mequiv./kg hemoglobin per h (between 120 and 200 min).

Effects of calcium. In order to test for possible involvement of a calcium-mediated effect on the K^+ channel we omitted the calcium from the medium and added 5 mM EGTA to chelate traces of calcium. Even in the absence of free calcium (Fig. 1) a potassium efflux and a sodium influx occurred after a lag period of 2 h. This K^+ efflux is significantly smaller than that in the presence of calcium, but there is no difference in the sodium permeability. Our attention in the next experiments is focussed on the calcium-independent permeability change, termed the 'ferricyanide effect'.

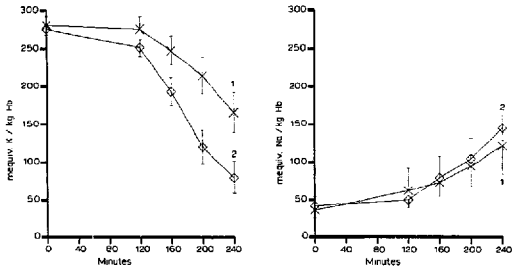

Fig. 1. Effect of calcium in ferricyanide experiments on K^+ and Na^+ content of red blood cells as a function of time. The incubation medium contained 150 mM NaCl, 20 mM Hepes, 5 mM ferricyanide, 2 mM adenosine, 0.2 mM IAA and either 5 mM EGTA (curve 1) or 0.5 mM calcium (curve 2). Bars indicate S.D. ($n = 4$).

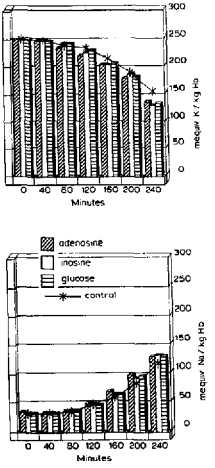

Fig. 2. Effect of different substrates on cation permeability in the ferricyanide experiment. The control medium contained 150 mM NaCl, 20 mM Hepes, 5 mM EGTA, 5 mM ferricyanide, 0.2 mM IAA at pH 7.4 (37 °C). Adenosine, inosine or glucose were added at a final concentration of 2 mM.

Requirements for the permeability increase

The next experiments will show which components are necessary for this 'ferricyanide effect'.

Ferricyanide. If ferricyanide is replaced by 5 mM ferrocyanide the K^+ and Na^+ fluxes do not differ from normal leak fluxes (data not shown). Since ferricyanide can act as an electron acceptor, the effectiveness of ferri- but not ferrocyanide suggests that oxidation of some cell or membrane component may be important in increasing the cation permeability. If sodium ferricyanide is used, the fluxes are the same as with potassium ferricyanide (data not shown). This is in contrast to the Gardos effect, where stimulation requires extracellular potassium.

Iodoacetate. The changes in potassium and sodium permeability will not occur if IAA is not present (data not shown). Hence both ferricyanide and IAA are necessary for the increase of K^+ and Na^+ fluxes in calcium-free medium. Each substance alone is without effect.

Metabolic substrates. Adenosine, inosine or glucose have only a very small effect on the permeability change (Fig. 2).

Comparison with the Gardos system and PCMBS

In the next series of experiments, we compare the ferricyanide effect with the Gardos effect, to see whether or not a common mechanism might be involved, with an altered Na^+/K^+ selectivity in the case of the ferricyanide effect. Also we compare the ferricyanide-induced increase in permeability with that caused by PCMBS. It has been proposed that ferricyanide might interact with exofacial SH groups [6]. PCMBS, which does bind to membrane SH groups, increases both Na^+ and K^+ permeability after a lag period [4,11], thus at least superficially resembling the ferricyanide effect (Fig. 1).

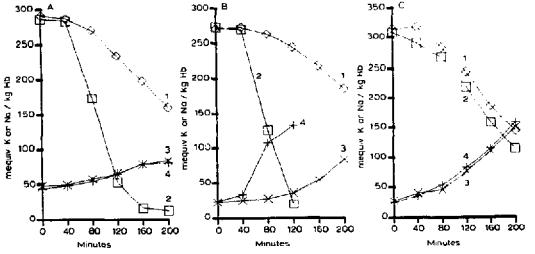

Fig. 3. Effect of 0.5 mM $NaVO_3$ in a Gardos-type (A), ferricyanide (B) and PCMBS (C) experiment. The Gardos-type experiment is run at 37 °C at pH 7.4 in 150 mM NaCl, 20 mM Hepes, 0.5 mM $CaCl_2$, 2 mM adenosine, 2.5 mM IAA. The ferricyanide-type experiment is run in 150 mM NaCl, 20 mM Hepes, 5 mM EGTA, 5 mM ferricyanide, 2 mM adenosine, 0.2 mM IAA at 37 °C (pH 7.4). The incubation medium in the PCMBS experiment contained 50 μM PCMBS, 150 mM NaCl, 20 mM Hepes, 5 mM EGTA (pH 7.4) at 37 °C. Curve 1, K^+ control; curve 2, $K^+ + NaVO_3$; curve 3, Na^+ control; curve 4, $Na^+ + NaVO_3$.

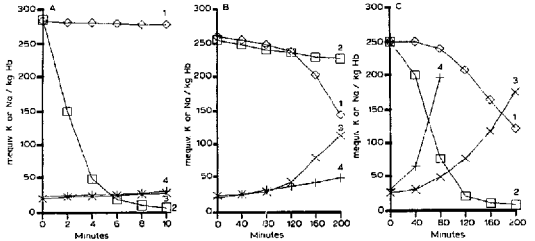

Fig. 4. Effect of 1 μM A23187 on Na^+ and K^+ content of red cells as a function of time in a Gardos-type (A), ferricyanide (B) and PCMBS (C) experiment. The experimental conditions are as given in the legend to Fig. 3.

Effects of vanadate. In the Gardos-type experiment (Fig. 3A) the addition of vanadate stimulates the K^+ permeability as demonstrated recently [12–14].

In the case of the ferricyanide effect, vanadate increases both potassium and sodium permeabilities (Fig. 3B). The effect of $NaVO_3$ is exerted inside the cell, because addition of the anion channel blocker H_2DIDS prevents this stimulation [9].

The addition of vanadate does not influence the cation permeability increase caused by PCMBS in the presence of 5 mM EGTA (Fig. 3C), in contrast to the Gardos-type and ferricyanide experiments

A23187. The addition of the calcium ionophore A23187 under the three different conditions has very different consequences: In the Gardos-type experiment the calcium ionophore increases intracellular free calcium [14]. The potassium efflux is extremely stimulated (notice the time axis in Fig. 4A) while the sodium flux remains unaffected. Under ferricyanide-effect conditions, the fluxes of both cations are blocked (Fig. 4B). Just the opposite is seen in the experiment with PCMBS: The calcium ionophore has a stimulatory effect on the permeability changes of potassium and sodium (Fig. 4C). These effects of A23187 cannot be attributed to free calcium, because 5 mmol/l EGTA are present in both experiments.

Atebrin. Since ferricyanide does not enter the cell, and since only ferricyanide and not ferrocyanide is effective in eliciting the permeability increase, we previously suggested [9] that a transmembrane dehydrogenase might be involved in the effect. Therefore, we tested atebrin, a dehydrogenase inhibitor [15], on the three systems. In the Gardos- and ferricyanide-type experiments an inhibition of the cation fluxes occurs (Fig. 5A and 5B) in contrast to the PCMBS experiment where atebrin causes an increase in cation permeability (Fig. 5C). Although the inhibition of the ferricyanide effect by atebrin might be taken as evidence in favor of dehydrogenase involvement, the effects on the other two systems demonstrates the lack of specificity of this

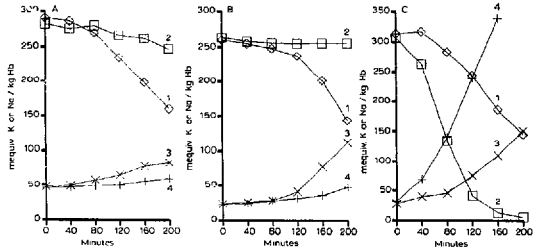

Fig. 5. Effect of 0.1 mM atebrin on potassium and sodium permeability in a Gardos-type (A), ferricyanide (B) and PCMBS (C) experiment. The experimental conditions are as given in the legend to Fig. 3.

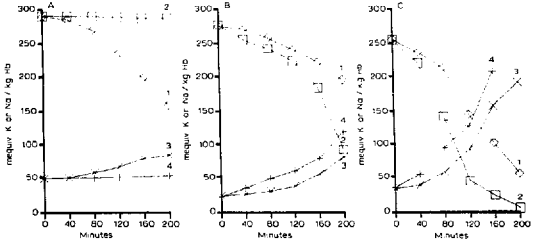

Fig. 6. Effect of 1 mM quinidine on cation permeability in a Gardos-type (A), ferricyanide (B) and PCMBS (C) experiment. The experimental conditions are as given in the legend to Fig. 3.

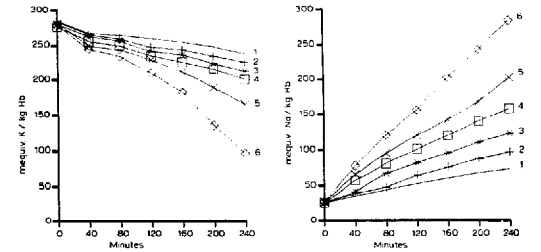

Fig. 7. A dose response for quinidine in the presence of 1 μM A23187 under ferricyanide experiment conditions (see legend to Fig. 3B). Curve 1, control; curve 2, 0.10 mM quinidine; curve 3, 0.25 mM quinidine; curve 4, 0.50 mM quinidine; curve 5, 0.75 mM quinidine; curve 6, 1.00 mM quinidine.

inhibitor. For example we have found that besides inhibiting the transmembrane NADH dehydrogenase by 40%, 0.1 mM atebrin reduces the probability of Ca^{2+}-induced K^+ channel opening by 20% [16]. Nevertheless, the differential effects of atebrin do provide further evidence that PCMBS-induced and ferricyanide-induced permeability increases involve different mechanisms.

Quinidine. Quinidine has been used as an inhibitor of the Ca^{2+}-activated potassium channel [1,2,13]. In contrast to the inhibitory action of quinidine in the Gardos experiment (Fig. 6A), there is an increase in K^+ and Na^+ permeability in the experiments with ferricyanide (Fig. 6B) and PCMBS (Fig. 6C).

Modulation of ion selectivity

Quinidine alone causes a slight increase (Fig. 6B). A23187 alone a complete block (Fig. 4B) of the ferricyanide-induced K^+ and Na^+ permeability. The combined action of both substances gives rise to an interesting phenomenon: The sodium influx increases at early times with little effect on the potassium efflux. A dose response of quinidine in the presence of A 23187 shows this effect in more detail (Fig. 7). Fig. 8 demonstrates that in the early time period between 40 and 80

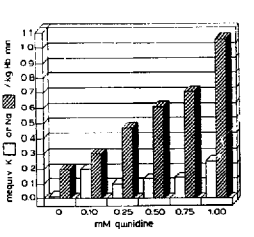

Fig. 8. Potassium and sodium flux of Fig. 7 in the time period of 40 to 80 min shown as a function of the quinidine concentration.

min concentrations of quinidine between 0.1 and 1 mM greatly stimulate the Na^+ flux while the K^+ flux remains nearly unchanged. No permeability changes occur when ferricyanide is omitted (results not shown).

Discussion

The combined action of ferricyanide and IAA produces an increase in potassium permeability. However, in contrast to the opinion of Passow and Gruner [5] that this is a highly specific effect on potassium permeability there is also a change in sodium permeability. The 'ferricyanide effect' is not related to the Gardos effect because the influence of calcium can be excluded by washing the freshly drawn red blood cells in a buffer containing EDTA and by measuring the fluxes in the presence of 5 mM EGTA, which prevents calcium entry into the cell. The sodium influx remains unchanged, while the potassium efflux is diminished slightly but significantly. The increase of the potassium flux in the presence of calcium may be the consequence of an additional Gardos effect.

In addition to the different effects on K^+ and Na^+ permeability, the ferricyanide effect and the Gardos effect differ in terms of their sensitivities to different agents (see Table I). In particular, A23187 and quinidine have opposite effects on these two processes.

The ferricyanide effect is also different from the nonspecific permeability change elicited by PCMBS. With the ferricyanide system, it is possible to change the sodium permeability separately from that of potassium. Also, A23187 and atebrin have opposite effects on the ferricyanide effect and the PCMBS effect (Table I). Thus, if external SH groups are involved in the ferricyanide effect, it seems unlikely that they are the same as those involved in the effect of PCMBS.

Heller et al. [17] have found that with 0.2 mM IAA, 0.5 mM vanadate and 5 mM ferricyanide (IVF), the membrane becomes leaky to small solutes such as chloride and erythritol. Under these conditions Na^+ and K^+ permeability are also increased (see Fig. 3B), and the cells begin to hemolyse [17]. These effects were interpreted as being due to oxidative damage to the membrane. The ferricyanide effect described here, in the absence of vanadate, seems to be very different from the effect described by Heller et al. [17]: In the first place, the IVF effect is very strongly enhanced if inosine is added instead of glucose, whereas the ferricyanide effect described here is very little affected by addition of inosine (Fig. 2). Secondly Heller et al. [17] found no increase in permeability to chloride and erythritol in the absence of vanadate. Thus, the ferricyanide effect described here does not involve formation of nonspecific channels of the type described by Heller et al. [17].

In summary, the data indicate that the ferricyanide effect is distinct from other mechanisms which increase red blood cell potassium permeability, such as the Gardos effect, the PCMBS effect, and the IVF effect. The mechanism of this permeability change is not yet known, but may involve some change in the redox state of the cell, since the electron acceptor ferricyanide, but not ferrocyanide produces the permeability change. The possible mechanism of the permeability change will form the subject of a subsequent paper.

Acknowledgements

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TABLE I

Changes of potassium efflux and sodium influx for different types of experiments in the presence of various additives

i, increase; d, decrease; 0, no effect; s, slight; –, not done.

	Gardos		Ferricyanide		PCMBS	
	K^+	Na^+	K^+	Na^+	K^+	Na^+
Control	i	si	i	i	i	i
+ adenosine	i	0	si	si	–	–
+ vanadate	i	0	i	i	0	0
+ A23187	i	0	d	d	i	i
+ atebrin	d	sd	d	d	i	i
+ quinidine	d	sd	i	i	i	i

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