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HYPERPOLARIZATION OF MOUSE SKELETAL MUSCLE PLASMA MEMBRANE INDUCED BY EXTRACELLULAR NADH

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Extracellularly applied NADH, but not NAD or NADPH, increases the resting membrane potential from −74.1 to −76.6 mV in freshly isolated muscles in the presence of K^+ in the incubation medium and from −64.6 to −72.9 mV in muscles equilibrated for 4–6 h in a K^+-free solution. The NADH-induced hyperpolarization is blocked by pretreatment of muscles with ouabain, and the inhibitors of plasma membrane NADH dehydrogenase (adriamycin, azide, PCMB, atebrine, DIDS and bleomycin). The effect of NADH is accompanied by the disappearance of NADH from the incubation medium and by decreased membrane resistance. We conclude that NADH hyperpolarization is due to the enhancement of passive membrane permeability, apparently for K^+, which might result from the conformational changes in the plasma membrane during the NADH dehydrogenase reaction. The possibility is discussed that NADH dehydrogenase mediates transport of K^+ out from the cell using a pathway connected with the transmembrane Na^+/K^+ pump.

Introduction

Fragmented plasma membranes from the brain, kidney, red blood cell and cardiac muscle contain a system that can transfer electron from NADH to cytochrome *c* [1] and/or, with a much higher efficacy, to ferricyanide [2]. NADH dehydrogenase in the liver and fat cell plasma membranes differs in its drug sensitivity from similar structures in mitochondrial membranes [3]. The physiological role of oxidoreducing reactions in the plasma membrane is not clear [4], although energization of active transport of cations [5] or amino acids [6,7] had been suggested. It has recently been shown that the redox activity of plasma membranes stimulates 'downhill' transport of monovalent cations according to their electrochemical gradients [8,9]. Such changes in transport may be accompanied by changes in the membrane potential which depends to a great extent on the permeability for potassium and on the concentration gradient of ions across the membrane. The muscle cell provides a good model for the concomitant estimation of membrane polarization, membrane permeability and membrane dehydrogenase activity. For this reason, we measured the oxidation capacity of intact as well as fragmented plasma membranes of skeletal muscle and compared it with the resting membrane potential recorded by intracellular microelectrodes. Evidence has been obtained that oxidation of NADH by intact muscle cell in the absence of arteficial electron acceptor is accompanied by membrane hyperpolarization and by decreased membrane resistance.

Abbreviations: PCMB, *p*-chloromercuribenzoate; DIDS, 4,4′-diisothiocyano-2,2′-disulfonic acid stilbene.

Methods and Material

Electrophysiological measurements of resting membrane potential and input membrane resistance. Experiments were performed on the hemidiaphragm of female white mice (20 ± 5 g body wt.). Muscles were isolated after decapitation and washed for 30 min up to 6 h continuously with oxygenated (95% O_2/5% CO_2) solution [10] of the following composition (in mmol · l^{-1}): Na^+, 149,8; K^+, 5.0; Ca^{2+}, 2.0; Mg^{2+}, 1.0; HCO_3^-, 12.8; $H_2PO_4^-$, 1.0; glucose, 11.0; pH 7.2, or solutions where K^+ was omitted. Muscles were mounted on a Sylgard-covered bottom of 5 ml chamber, and resting membrane potentials were recorded by the standard electrophysiological technique from superficial muscle fibres with intracellular glass microelectrodes filled with 3 mol · l^{-1} KCl (resistance 10–20 MΩ).

The effective input membrane resistance (R_e) of muscle fibres was calculated from the changes of the membrane potential in response to rectangular pulses of 8–10 ms duration passed through a second intracellular microelectrode (2 mol · l^{-1} potassium citrate) inserted into the same muscle fibre some 20 μm apart [11].

Experiments were performed at room temperature of 20 ± 2 °C.

Measurement of NADH dehydrogenase in intact skeletal muscles. The rate of NADH oxidation was determined by continuous recording of the disappearance of NADH from the incubation medium in the presence of a piece (usually a quarter) of the mouse diaphragm. The tendon of the muscle was tied to a thread and the muscle was transported into a cuvette containing 2 ml K^+-free solution with NADH, the content of which was measured in a Perkin-Elmer fluorimeter (excitation, 366 nm; emission, 455 nm) [12]. The reaction was started by immersion of the muscle into the medium and after 2–5 min delay, the reaction became linear for at least 15 min, but only the first 10 min were taken for the calculation of activity. Care was taken to keep the muscle in the upper part of the cuvette to avoid passage of the beam through the muscle. Initial concentration of NADH, $1 \cdot 10^{-5}$ mol · l^{-1} in the reaction medium was found to be optimal for the measurement of NADH -dehydrogenase activity of intact muscle. On the basis of higher NADH concentration ($1 \cdot 10^{-4}$ mol · l^{-1}), no NADH oxidation could yet be seen and when NADH was used in a concentration $1 \cdot 10^{-6}$ mol · l^{-1}, no NADH oxidation could be detected.

Measurement of NADH : ferricyanide oxidoreductase in membrane fractions of mouse skeletal muscle. Plasma membranes were prepared from mouse skeletal muscles (m. gastrocnemius) according to the method of Matsui and Schwartz [13]. The standard assay system consisted of $5 \cdot 10^{-4}$ mol · l^{-1} ferricyanide, $4.8 \cdot 10^{-4}$ mol · l^{-1} NADH, inhibitors as indicated, 1 ml sodium phosphate buffer (100 mmol · l^{-1}) (pH 7.4) and an appropriate amount of membrane fractions (30 μg protein) [14]. NADH : ferricyanide oxidoreductase was measured as a decrease of absorbance at 340 nm on a Beckman spectrophotometer. The inhibitors and the tested compounds were added to the membranes before adding ferricyanide which started the reaction.

Chemicals. The following chemicals were from sources as indicated: NADH (disodium salt), NAD (disodium salt) and NADPH (tetrasodium salt) from Boehringer; atebrine (quinacrine hydrochloride), Ca^{2+} ionophore A23187, DIDS, and rotenone from Sigma; ouabain from Fluka; adriamycin from Adriablastina, Farm Italia; and bleomycin from Nippon Kayaku.

Results

Effect of extracellularly applied NADH on the resting membrane potential

The resting membrane potentials of mouse diaphragm muscle fibres were measured before and 10–20 min after application of NADH into the bath with or without potassium (Fig. 1). In solutions containing 5 mmol · l^{-1} K^+, a hyperpolarization of about 2.5 mV was observed at concentrations of $1 \cdot 10^{-6}$ mol · l^{-1} NADH. When potassium was omitted and muscles were equilibrated in K^+-free solutions for 4–6 h, the resting membrane potential decreased from -74.1 ± 0.8 to -64.6 ± 0.8 mV and NADH induced markedly higher hyperpolarization which reached 8.3 mV with $1 \cdot 10^{-6}$ and $1 \cdot 10^{-5}$ mol · l^{-1} NADH.

Neither prolonged incubation of muscles for 4–6 h in a normal solution (resting membrane potential $= -68.4 \pm$ mV) nor brief incubation in

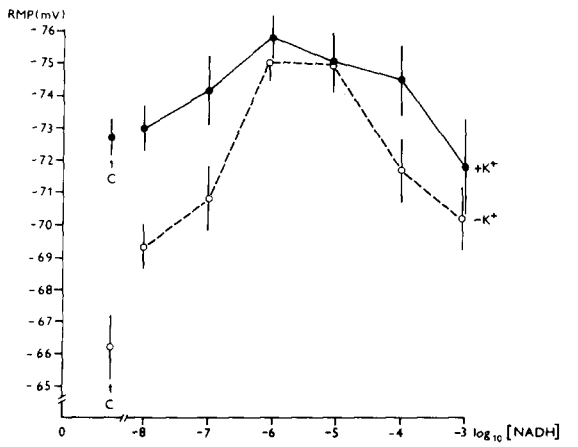

Fig. 1. Resting membrane potential (RMP) after application of NADH to the bath. Measurements were performed on freshly isolated muscles in 5 mmol·l^{-1} K^+ incubation solution ($+K^+$, full line) and/or on Na^+-loaded muscles in K^+-free solution ($-K^+$, dotted line). Each concentration of NADH was tested separately on 3–6 muscles where 15–20 fibres were recorded in each muscle before (C, indicated by arrows) and 10 min after NADH application. Values are given as the means ± S.E.

a K^+-free solution (resting membrane potential = -90.7 ± 1.6 mV) potentiated the response of the membrane to NADH, and the hyperpolarization did not exceed 3 mV in either case.

NAD and NADPH added in concentrations of $1 \cdot 10^{-5}$ mol·l^{-1} to the muscles equilibrated for 4–6 h in K^+-free bath casued no hyperpolarization within 10 min after application. The resting membrane potential was -65.1 ± 0.4 and -66.0 ± 1.3 mV before and after NAD, respectively, and -61.3 ± 0.4 and -58.3 ± 1.9 before and after NADPH, respectively. These data indicate that the hyperpolarizing effect is specific for NADH.

Regulation of NADH-induced hyperpolarization

To test the possibility that the hyperpolarization observed after NADH is coupled to NADH oxidation in the plasma membrane, the sensitivity of NADH hyperpolarization to the inhibitors of plasma membrane NADH dehydrogenase such as atebrine, azide [3], adriamycin [15], *p*-chloromercuribenzoate (PCMB) [14], DIDS and bleomycin (Table IV) was tested. All drugs were added to the muscles 10–20 min before $1 \cdot 10^{-5}$ mol·l^{-1} NADH.

NADH hyperpolarization was entirely blocked by DIDS ($1 \cdot 10^{-5}$ mol·l^{-1}) and by adriamycin ($1 \cdot 10^{-4}$ mol·l^{-1}), and was lowered by azide ($1 \cdot 10^{-3}$ mol·l^{-1}), PCMB ($1 \cdot 10^{-5}$ mol·l^{-1}), atebrine ($1 \cdot 10^{-4}$ mol·l^{-1}) and bleomycin ($1 \cdot$

TABLE I

THE EFFECT OF NADH DEHYDROGENASE INHIBITORS ON RESTING MEMBRANE POTENTIAL AND ON NADH HYPERPOLARIZATION

The experiments were performed on muscles equilibrated for 4–6 h in a K^+-free medium. NADH was applied to muscles pretreated for 10 min with the tested drugs. Resting membrane potentials were measured before and after application of the drugs (difference Δ_1; negative values represent hyperpolarization; positive values indicate depolarization) and 10 min after NADH $1 \cdot 10^{-5}$ mol·l^{-1} (difference Δ_2). Mean resting membrane potential was calculated from measurements on n muscles where 15–20 impailments were performed in each muscle. S.E. (not indicated) is in the range of 0.5–1.9 mV. Differences are significant at $P < 0.01$ (*) and $P < 0.05$ (**).

Drug (mol·l^{-1})	Resting membrane potential (mV)					
	Control	After drug	Δ_1	After NADH	Δ_2	n
None	−67.6	–	–	−75.8	−8.3 *	20
Rotenone (10^{-5})	−59.9	−62.6	−2.7	−69.4	−6.8 *	4
Atebrine (10^{-4})	−62.6	−68.5	−5.9 *	−73.2	−4.7 *	4
Azide (10^{-3})	−62.6	−59.3	+3.3 **	−62.5	−3.2 **	4
Adriamycin (10^{-4})	−63.9	−64.7	−0.8	−65.3	−0.6	3
PCMB (10^{-5})	−66.8	−65.8	+1.0	−70.2	−4.4 **	3
DIDS (10^{-5})	−65.8	−65.4	+0.4	−62.7	+2.7	3
Bleomycin (10^{-6})	−64.4	−64.9	−0.5	−70.4	−5.5 *	3
A23187 (10^{-5})	−65.8	−64.7	+1.1	−67.9	−3.2 **	6
Ouabain (10^{-4})	−66.8	−64.1	+2.7 **	−65.3	−1.2	4

10^{-6} mol · l^{-1}) by 62, 48, 44 and 34%, respectively. Rotenone, a well-known inhibitor of mitochondrial NADH dehydrogenase, in the concentration $1 \cdot 10^{-5}$ mol · l^{-1}, lowered NADH hyperpolarization by only 18% (Table I).

Ouabain, an inhibitor of ($Na^+ + K^+$)-ATPase [16], in the concentration of $1 \cdot 10^{-4}$ mol · l^{-1}, was also found to inhibit the NADH effect by 85% (Table I).

The effect of NADH on transmembranous K^+ transport was reported to be enhanced by the Ca^{2+}-ionophore A23187 [17], but in our case NADH-induced hyperpolarization was lowered by ionophore A23187 by 61%.

When applied alone, some of the compounds tested influenced the resting membrane potential. Azide and ouabain depolarized the membrane by about 3 mV, atebrine hyperpolarized the membrane by about 6 mV (Table I), but there was no correlation between the membrane potential changes and the effect on NADH hyperpolarization.

Comparison of NADH hyperpolarization with K^+-hyperpolarization of Na^+-loaded muscles

Incubation of the mouse diaphragm for 4–6 h in K^+-free solution results in a decrease of resting membrane potential by 8–10 mV, accompanied by an increase in intracellular Na^+ concentration (from about 15 to 30 mmol · l^{-1}) [18]. Application of potassium to the bath with such so-called 'Na^+-loaded' muscles induces ouabain-sensitive hyperpolarization (K^+ hyperpolarization) which represents a maximal electrogenic effect of the Na^+/K^+ pump [19]. As we described previously (see Fig. 1), also NADH produced significant hyperpolarization only when applied to the muscles preincubated for 4–6 h in zero potassium. To know whether the same hyperpolarizing mechanism is involved, we compared the individual effects of K^+ and NADH with the effect of both K^+ and NADH applied together. 5 mmol · l^{-1} K^+ induces an increase of resting membrane potential by about 12 mV (from -65.3 ± 0.8 to -77.4 ± 1.0 mV, $n = 32$ and 7, respectively), $1 \cdot 10^{-5}$ mol · l^{-1} NADH hyperpolarized by about 9 mV (to -74.6 ± 1.8 mV, $n = 10$) but $1 \cdot 10^{-5}$ mol · l^{-1} NADH plus 5 mmol · l^{-1} K^+ applied together hyperpolarized again by only 12 mV (to -77.8 ± 1.6 mV, $n = 12$). This indicates that the effects of NADH and K^+ on Na^+-loaded muscles are not additive.

Membrane resistance changes during NADH hyperpolarization

Membrane resistance is reciprocally related to the membrane conductance which determines the membrane permeability and the value of resting membrane potential [20]. A good measure of passive membrane resistance is the effective input membrane resistance (R_e).

Muscles equilibrated for 4–6 h in a K^+-free solution had higher R_e values than freshly isolated muscles incubated in a K^+-containing medium (Table II). Within 10–30 min after application of NADH, the membrane resistance was lowered in freshly isolated and Na^+-loaded muscles by 7 and 29%, respectively (Fig. 2 and Table II). The effect of NADH on membrane resistance was not inhibited by ouabain; ouabain itself decreased R_e by 10%, probably by increasing the sodium permeability [21]. During activation of the electrogenic pump by application of 5 mmol · l^{-1} K^+ to Na^+-loaded muscles in a K^+-free solution, no decrease but rather an increase of R_e by about 10% (Table II) was observed.

TABLE II

MEMBRANE RESISTANCE CHANGES DURING NADH-INDUCED HYPERPOLARIZATION

The effective input membrane resistance (R_e) was measured in mouse diaphragm muscles incubated for about 30 min in a normal solution containing 5 mmol · l^{-1} K^+ ($+K^+$) and/or in muscles equilibrated for 4–6 h in a K^+-free medium ($-K^+$). The effects of NADH, $1 \cdot 10^{-5}$ mol · l^{-1}, ouabain, $1 \cdot 10^{-4}$ mol · l^{-1}, and 5 mmol · l^{-1} K^+ were measured within 10–30 min after application. Values are given as a mean ± S.E. calculated from measurements on n muscles where 10–20 recordings were made in each muscle. The differences between control and experimental values are significant at $P < 0.01$ (*) and $P < 0.05$ (**).

	R_e ($\times 10^5$ Ω)			
	$+K^+$	n	$-K^+$	n
Control	6.89 ± 0.32	9	9.35 ± 0.77	12
NADH	6.45 ± 0.49	2	6.68 ± 0.16 *	3
NADH + ouabain	–	–	6.96 ± 0.45 *	2
Ouabain	–	–	8.50 ± 0.42	4
5 mol · l^{-1} K^+	–	–	10.93 ± 0.22 **	3

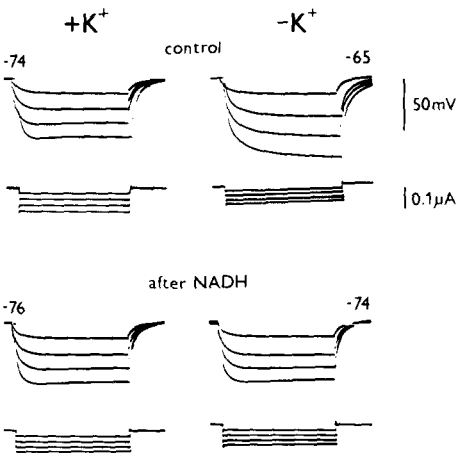

Fig. 2. Intracellular records of typical responses of membrane potential to hyperpolarizing current pulses in freshly isolated muscles incubated in a normal solution ($+K^+$) and in Na^+-loaded muscles immersed in a K^+-free solution ($-K^+$). The curves show responses before and after NADH (10^{-5} mol·l^{-1}) application. The fibres were maintained at the resting membrane potential levels indicated by the numbers.

Oxidation of NADH by the skeletal muscle

It has recently been found that the NADH : ferricyanide oxidoreductase reaction can also proceed on the external surface of intact mouse ascites tumor cells but no endogenous dehydrogenase activity was found in the absence of ferricyanide [22]. Using NADH in a concentration $1 \cdot 10^{-5}$ mol·l^{-1}, which is 10-times lower than that used by Cherry et al. [22], and using a more sensitive fluorimetry assay, we were able to detect the oxidation of extracellularly added NADH by intact skeletal muscle in the absence of any added electron acceptor. The activity of NADH dehydrogenase in diaphragm was 5.51 ± 0.48 ($n = 5$ muscles) nmol oxidized/min per mg protein. It was inhibited by 42% with $1 \cdot 10^{-4}$ mol·l^{-1} atebrine (to 3.18 ± 0.7 nmol NADH, significant at $P < 0.01$) but ony by 14% with $1 \cdot 10^{-5}$ mol·l^{-1} rotenone (nonsignificant). No appreciable inhibition (8%) was found in the presence of ouabain ($1 \cdot 10^{-4}$ mol·l^{-1}).

NADH : ferricyanide oxidoreductase in membrane fractions

Remarkably higher activity of the enzyme oxidizing NADH could be obtained in membrane fractions in the presence of ferricyanide [2]. In our case, NADH : ferricyanide oxidoreductase activity of muscle plasma membrane fragments was about 20 nmol oxidized NADH/min per mg protein. The effect of inhibitors and some membrane-active drugs on NADH : ferricyanide oxidoreductase is shown in Table III. The most effective inhibition was observed with bleomycin ($1 \cdot 10^{-6}$ mol·l^{-1}), DIDS ($1 \cdot 10^{-5}$ mol·l^{-1}) and PCMB ($1 \cdot 10^{-5}$ mol·l^{-1}) which lowered the activity of enzyme by 70, 60 and 50%, respectively. Inhibition by 80% could be obtained with atebrine, adriamycin, and azide but in higher concentrations ($1 \cdot 10^{-3}$ to $1 \cdot 10^{-1}$ mol·l^{-1}). Ouabain ($1 \cdot 10^{-4}$ mol·l^{-1}) lowered NADH : ferricyanide oxidoreductase activity by 35%. Stimulation of the enzyme activity by about 70% was observed in the presence of Ca^{2+}-ionophore A23187 (Tab. III).

TABLE III

THE EFFECT OF INHIBITORS ON NADH : FERRICYANIDE OXIDOREDUCTASE IN MUSCLE PLASMA MEMBRANE FRAGMENTS

Activity of NADH : ferricyanide oxidoreductase is expressed in % of control values (=100%). All tested compounds were added into the reaction medium in the indicated concentrations 10 min before ferricyanide to start the reaction. Values represent the mean ± S.E. of n enzymatic measurements. All changes in the activity before and after application of drugs are significant at $P < 0.01$.

Drug	Conn. (mol·l^{-1})	Activity (%)	n
None		100 ± 3	20
Rotenone	10^{-5}	89 ± 5	13
Atebrine	10^{-4}	72 ± 3	5
	10^{-3}	21 ± 8	5
Azide	10^{-3}	92 ± 3	2
	10^{-1}	20 ± 4	8
PCMB	10^{-5}	50 ± 4	4
Adriamycin	10^{-4}	79 ± 4	5
	10^{-3}	21 ± 5	5
Bleomycin	10^{-6}	30 ± 5	7
DIDS	10^{-5}	30 ± 4	5
A23187	$5 \cdot 10^{-6}$	169 ± 7	5
Ouabain	10^{-4}	65 ± 8	3

Discussion

It has been demonstrated that when NADH is applied extracellularly, it increases the resting membrane potential of mouse skeletal muscle

fibres. This hyperpolarizing effect of NADH is potentiated by the lowering of basal resting membrane potential and by the absence of K^+ in the solution and can be observed within 10 min after application of NADH in the concentration range of $1 \cdot 10^{-8}$ to $1 \cdot 10^{-4}$ mol $\cdot l^{-1}$. In order to understand the effect of NADH, two questions should be answered: (1) What is the membrane mechanism underlying the NADH-induced hyperpolarization? (2) Does oxidation of NADH play a role in this hyperpolarizing mechanism?

Hyperpolarization induced by NADH could be mediated by the electrogenic pump catalyzed by plasma membrane ATPase. The following findings seem to be in favour of this possibility: The effect of NADH is blocked by pretreatment with ouabain, higher values of NADH hyperpolarization are obtained in muscles which have lower membrane potential (apparently because of a higher level of intracellular sodium [18], and NADH hyperpolarization is not additive to K^+-induced hyperpolarization of Na^+-loaded muscles which is a measure of the maximal electrogenic effect of the Na^+/K^+ pump [19]. Nevertheless, severe objections may be raised against the involvement of active membrane transport in the mechanism of NADH hyperpolarization. NADH can hyperpolarize the plasma membrane of Na^+-loaded muscles in the absence of extracellular K^+, but, up to now, stimulation of Na^+-Na^+ exchange via $(Na^+ + Na^+)$-ATPase is believed to be purely electroneutral [23,24]; NADH hyperpolarization is accompanied by a decrease of membrane resistance, while stimulation of the electrogenic Na^+/K^+ pump in Na^+-loaded muscles by potassium increases membrane resistance.

The mechanism of NADH hyperpolarization must, therefore, involved an increase of membrane permeability. One can exclude the possibility that the permeability for sodium is enhanced, since this would depolarize the membrane [20]. Increased membrane permeability for Cl^- could be also theoretically associated with depolarization [25]. Enhancement of passive membrane permeability for K^+ is the only mechanism which may account for the NADH hyperpolarization. Actually, stimulation of K^+ transport across the membrane was found in human erythrocytes treated with the arteficial electron donor system, ascorbate plus phenazine methosulphate [8], or with phenazine methosulphate only [9]. It has even been suggested that the effect of electron donors on K^+ transport is a 'Gardos effect', i.e., Ca^{2+}-dependent K^+-channel activation, since it was enhanced by Ca^{2+}-ionophore A23187 [8]. We did not find any increase of the NADH effect in intact muscles preincubated with ionophore A23187. On the other hand, the NADH : ferricyanide oxidoreductase reaction itself was stimulated by calcium ionophore.

The second question which should be answered in this paper is whether the enzymatic reaction plays a role in the mechanism underlying NADH hyperpolarization. In most studies on the activity of plasma membrane oxidoreductase, natural as well as arteficial electron donors are suggested to be accessible only from the cytoplasmic side of the membrnae [4], but it has been found that dehydrogenase reaction can also proceed, in the presence of ferricyanide, on the external surface of the membrane [22]. We found that extracellularly added NADH can be oxidized by intact skeletal muscle in the absence of an arteficially added oxidizing agent. Further, NADH but not NAD or NADPH increases the membrane potential and this effect was inhibited or was lowered by 40–60% with the inhibitors of NADH dehydrogenase (the natural electron acceptor is unknown) and NADH : ferricyanide oxidoreductase (ferricyanide is arteficially added as the electron acceptor) such as DIDS, adriamycin, PCMB, azide, atebrine and bleomycin.

We assume that the oxidoreducing reaction in the membrane induces conformational changes which could increase membrane permeability and enhance the number of passive resting K^+ channels in the membrane. It is possible, for example, that potassium leakage from the cell results from the damage of membrane lipids induced by oxygen radicals which might be catalyzed by the NADH dehydrogenase reaction [9]. Furthermore, it is feasible that the redox state of some membrane macromolecule which regulates the membrane permeability for K^+ is shifted during the redox reaction in the membrane. The ouabain sensitivity of the NADH-induced membrane potential changes implies that $(Na^+ + K^+)$-ATPase could be such a macromolecule. Since Skou and Esman [26] showed that the (intracellularly) protonated form of $(Na^+$

$+ K^+$)-ATPase macromolecule has a higher affinity for extracellular K^+, while the deprotonated form prefers Na^+, it could be speculated that ($Na^+ + K^+$)-ATPase protonated extracellularly by NADH would bind intracellular K^+ and release it into the extracellular medium. A similar reversion of the pump has already been suggested as a mechanism of K^+-K^+ exchange observed in red blood cells [27].

If the forward activation of the Na^+/K^+ pump increases membrane resistance (Table II), its backward stimulation might be associated with the decrease of membrane resistance.

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