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Chloride Cotransport in the Membrane of Earthworm Body Wall Muscles

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Summary

The resting membrane potential (V_m) of isolated somatic longitudinal muscles of the earthworm *Lumbricus terrestris* was studied by glass microelectrodes. The inhibition of chloride permeability by low pH did not affect V_m of the muscle fibers in isolated somatic longitudinal muscles of the earthworm *Lumbricus terrestris* which was –48.7 mV (inside negative) at pH 7.3 and –49.1 at pH 5.6. On the other hand, bathing the muscles in Cl^- and Na^+-free solutions, or application of the chloride transporter inhibitor furosemide and Na^+-K^+-ATPase inhibitor ouabain depolarized the V_m by 3-5 mV. The effects of a Cl^- -free solution and ouabain were not additive. This demonstrates relatively small contribution of equilibrium potential for Cl^- to the resting membrane potential and electrogenic effect of Na^+K^+-ATPase which is dependent on the supply of Na^+_i ions by furosemide-sensitive and Cl^-_e- and Na^+_e-dependent electroneutral transport (most probably $Na^+K^+Cl^-$ cotransport).

Key words

Earthworm • Resting membrane potential • Na^+-K^+-ATPase • Ouabain • Furosemide • Chloride transporter

Introduction

The resting membrane potential (V_m) of skeletal muscle fiber can be regulated by several mechanisms (Siegenbeek van Heukelom *et al.* 1994): the electrochemical potential (Edwards and Vyskočil 1984), ionic permeability (see eg. Shabunova and Vyskočil 1982, Giniatullin *et al.* 1999), pH (Volkov 1983), ionic pumps such as Na^+-K^+-ATPase (Kernan 1962, Vyskočil *et al.* 1995, Volkov *et al.* 2000) and the furosemide-sensitive Cl-transporter (Edwards and Vyskočil 1984, Urazaev *et al.* 1998a, 1999, 2002). V_m is also controlled by an osmotic state of the cell (Edwards 1982, Lang *et al.* 1995), hormones (Zemková *et al.* 1982, Adámek *et al.* 1996, 2002, Schutzner *et al.* 1999) and by long-lasting transmitter release regulated by second messengers, nitric oxide (Urazaev *et al.* 1998b, 1999, 2002, Mukhtarov *et al.* 1999, Nikolsky *et al.* 1999), and presynaptic autoreceptors (Bukharaeva *et al.* 1999, 2000).

The extent to which the Na^+-K^+ pump contributes to V_m in somatic muscles of the developmentally important phyllum *Annelidae* (Chang

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1969, Walker *et al.* 1993) was shown in our previous study (Volkov *et al.* 2000) where we demonstrated that this pump can hyperpolarize the muscle fiber membrane more effectively than in frog skeletal muscles when activated by extracellular K^+. In the present report we demonstrate pharmacologically the role of another electrogenic ion, Cl^- (Volkov *et al.* 2000) in earthworm muscles, and the existence of a chloride membrane transporter.

Methods

Experiments were performed on isolated neuromuscular preparations of longitudinal somatic muscles of the earthworm *Lumbricus terrestris* (Drewes and Pax 1974). Strips of the earthworm body wall muscle, approximately 10 segments in length, were prepared, from which the nerve cord and viscera had been removed. The resting membrane potential was measured by impaling 20 or more muscle fibers with glass microelectrodes (2.5 mol/l KCl, 7-15 MΩ) during a 5 min period before, and another 20 or more fibers 5-10 min after the addition of $1x10^{-4}$ mol/l ouabain (Kernan 1962, Zemková *et al.* 1982) or $1x10^{-4}$ mol/l furosemide (Sigma, USA) to the medium. The difference between the mean resting membrane potentials under these two conditions is generally considered to be due to the electrogenic activity of the sodium pump. The earthworm modified Drewes-Pax solution (Drewes and Pax 1974) contained (mM): NaCl 163; KCl 4; $CaCl_2$ 6; sucrose 167; Tris 2; pH 7.2-7.4, unless otherwise stated (Table 1). In each experimental group, 4-6 animals were used.

Microcal Origin, version 3.5, (Microcal Software, Inc. 1991-1994) was used for statistical analyses. Parametric analysis of variance (ANOVA) of the experimental groups versus the control group was made by multiple comparisons using the Bonferroni t-test. Throughout the text, statistically significant differences between the mean ± S.E.M. of two groups are indicated at the given level of probability P.

Table 1. Ionic composition of solutions (in mmol/l).

	Na^+	K^+	Ca^{2+}	TRIS	Cl^-	SO_4^{2-}	NO_3	Sucrose	Osmolarity mosmol/l	Ionic strength mmol/l
Control	163	4	6	2	93	43	-	167	478	229
Cl^--free	162	4	6	2	-	44	-	170	478	229
Cl^--free	162	4	6	2	-	-	90	170	478	229
Na^+-free	-	4	6	225	205	-	-	38	478	229

pH 7.2-7.4 and 5.6 were adjusted by titration

Results

The resting membrane potential (V_m) of earthworm muscle fibers (n = 400, 8 muscles) was –48.7±0.6 mV (inside negative) (Volkov *et al.* 2000) in control solution. Substitution of sulphates or nitrates for chlorides led to 4 mV depolarization, with no difference between substitutes. Depolarization was also seen in Na^+-free medium (Table 2) which was similar to that in Cl^--free solutions. On the other hand, lowering the pH to 5.6, which decreases the membrane Cl^- permeability (Eisenberg and Gage 1969, Volkov *et al.* 1987) had no effect on V_m. When muscles were incubated with the Na^+-K^+-ATPase antagonist, ouabain ($1x10^{-4}$ mol/l) for 5-10 min or furosemide, the blocker of electroneutral Na^+, K^+, $2Cl^-$ symport ($1x10^{-4}$ mol/l), the muscle fibers were depolarized to an extent similar to that seen in Na^+- and Cl^--free media (Table 2). Subsequent application of ouabain or furosemide to muscles bathed in Na^+- or Cl^--free media did not induce membrane depolarization. Thus, the effects were not additive (Table 2).

The present experiments show that removal of Cl^- and Na^+ leads to depolarization of earthworm muscle fibers, whereas the inhibition of passive chloride permeability by low pH does not change the V_m. Passive chloride permeability is a necessary prerequisite for the Nernst contribution of this ion to the V_m. If no change of V_m is observed after acidification which can close the

Cl channels, it means that the participation of Cl ions in V_m is negligible, if at all (Hodgkin and Horowicz 1959, Dulhunty 1978). The small effect of complete chloride removal could more probably support the idea that resting chloride conductance is much lower than the potassium conductance and that its further blockade could not cause a further change of membrane potential. Interestingly, the absence of the effect of pH changes also indicates that muscle membrane is not permeable for protons. On the other hand, the depolarization can be ascribed to some kind of electrogenic transport which hyperpolarizes the membrane and is abolished in Cl^-- and Na^+-free solutions

In many of vertebrate cell membranes, including those of muscle fibers, the membrane has a furosemide-sensitive Na^+, K^+, $2Cl^-$ symport (e.g. Haas *et al.* 1982, Aickin *et al.* 1989, Grege *et al.* 1983, Altamirano and Russel 1987). This symport is, however, electroneutral and its equilibrium is given by products of ions on both sides of the membrane $[Na]_i [K]_i [Cl]_i^2 = [Na]_o [K]_o [Cl]_o^2$ (Haas *et al.* 1982). It usually runs in "inward" direction being driven by the gradient of sodium and chloride ions. Accumulation of potassium ions inside the cell due to Na^+,K^+-ATPase or Na^+, K^+, $2Cl^-$ symport can by itself cause a Nernstian hyperpolarization. Na^+ permanently enters the fiber and is extruded outside by electrogenic Na^+,K^+-ATPase, i.e. by the process which also causes a hyperpolarization of V_m due to uneven number of transported positive charges. It is obvious that when no sodium is present outside, the intracellular Na^+ is rapidly pumped out and the Na^+K^+-ATPase is inhibited by the lack of sodium inside the fiber. This causes depolarization, which should be the same as after direct pump inhibition by ouabain (Table 2). The inhibition of the electrogenic sodium pump can also be caused by Cl^--free medium, because the Na^+, K^+, $2Cl^-$ symport directed inwardly is inhibited and no Na^+ is transported into the cell. One can even speculate that the application of Cl^--free solution could cause not only inhibition of the co-transport but also the reversion of neutral co-transport which then transports sodium and potassium outside the cell. Anyhow, the depletion of intracellular sodium is followed by the inhibition of electrogenic effect of Na^+K^+-ATPase and can be considered as a main cause of depolarization. The decrease of intracellular potassium which could be caused either by reversion of Na^+, K^+, $2Cl^-$ symport or by inhibition of Na^+K^+-ATPase can also cause depolarization, but this Nernstian influence of internal potassium decrease by several mmol/l is small.

Table 2. Resting membrane potential V_m of earthworm longitudinal muscles bathed in different solutions.

Solutions	**V_m (mV, inside negative)**	**n**
Control medium pH 7.2-7.4	48.7±0.6	400
Control medium pH 5.6	49.1±0.9	80
Cl^- -free medium	45.1±0.8*	202
Na^+-free medium	43.5±1.3*	80
Standard medium + ouabain	43.1±0.9*	180
Cl^--free medium + ouabain	42.3±1.0*	80
Standard medium + furosemide	43.5±1.0*	80

Mean ± S.E.M. are given. Ouabain and furosemide were applied in the concentration of $1x10^{-4}$ mol/l. n = number of muscle fibers. Asterisks indicate values that are significantly different from Control ($p<0.05$).

Discussion

The application of Na^+-free or Cl^--free solutions causes a depolarization of V_m. The resting membrane potential is also depolarized to the same level, when the sodium pump is blocked by ouabain and there is no Na^+ transport across the muscle fiber membrane. This ouabain-induced depolarization of earthworm muscle fibers has already been reported (Volkov *et al.* 2000) and was interpreted to be due to the elimination of electrogenic Na^+-K^+ ATPase activity.

In contrast, removal of chlorides from the bathing medium or inhibition of Na^+, K^+, $2Cl^-$ symport by furosemide does not substantially affect V_m in cold blooded vertebrates or mammals. This situation dramatically changes after motor denervation. Nerve section in vertebrates affects the activity of the ion transport systems responsible for the electrical properties of the sarcolemma, and the transmembrane ionic gradients and permeabilities (McArdle 1983, Shabunova and Vyskočil 1982). The first observed change after nerve section is a depolarization of the V_m (Albuquerque *et al.* 1971, Lorkovic and Tomanek 1977, Shabunova and Vyskočil 1982, Švandová *et al.* 2001) by about 8-10 mV (10-12 % of the control V_m) which occurs already within four hours of denervation (Bray *et al.* 1976, 1982, Urazaev *et al.* 1995, Urazaev *et al.* 1997, 1998a). We have shown that, to a great extent, furosemide prevents

this early postdenervation depolarization which is apparently caused by inward-directed, furosemide-sensitive chloride transport (Urazaev *et al.* 1997, 1999). In this case, passive V_m is higher than the symport-driven new chloride equilibrium.

In other words, inactivation of Cl^- transport leads to a depolarization of earthworm muscle fibers, but to a hyperpolarization (also seen as a prevention of depolarization) of denervated mammalian muscles. Because the concomitant presence of all three ions (Na^+ and Cl^- outside the cell and K^+ inside) is necessary for the proper functioning of the Na^+, K^+, $2Cl^-$ co-transport, it is clear that inhibition of the primary antiport of Na^+ and K^+ by ouabain stops the hyperpolarizing effect of chloride symport.

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