Electrical bistability of skeletal muscle membrane
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Chapter four
Bistable behaviour of muscle membrane potential

Effects of chloride transport on bistable behaviour of the membrane potential in mouse skeletal muscle

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The lumbrocal skeletal muscle fibres of mice exhibited electrically bistable behaviour due to the nonlinear properties of the inwardly rectifying potassium conductance. When the membrane potential ($V_m$) was measured continuously using intracellular microelectrodes, either a depolarization or a hyperpolarization was observed following reduction of the extracellular potassium concentration ($K^+$) from 5.7 mM to values in the range 0.76–3.8 mM, and $V_m$ showed hysteresis when $K^+$ was slowly decreased and then increased within this range. Hypertonicity caused membrane depolarization by enhancing chloride import through the Na$^+-K^+-2Cl^-$ cotransporter and altered the bistable behaviour of the muscle fibres. Addition of bumetanide, a potent inhibitor of the Na$^+-K^+-2Cl^-$ cotransporter, and of anthracene-9-carboxylic acid, a blocker of chloride channels, caused membrane hyperpolarization particularly under hypertonic conditions, and also altered the bistable behaviour of the cells. Hysteresis loops shifted with hypertonicity to higher $K^+$ values and with bumetanide to lower values. The addition of 80 µM BaCl$_2$, or temperature reduction from 35 to 27°C induced a depolarization of cells that were originally hyperpolarized. In the $K^+$ range of 5.7–22.8 mM, cells in isotonic media (289 mmol kg$^{-1}$) responded nearly Nernstianly to $K^+$ reduction, i.e. 50 mV per decade; in hypertonic media this dependence was reduced to 36 mV per decade (319 mmol kg$^{-1}$) or to 31 mV per decade (340 mmol kg$^{-1}$). Our data can explain apparent discrepancies in $A_{V_m}$ found in the literature. We conclude that chloride import through the Na$^+-K^+-2Cl^-$ cotransporter and export through Cl$^-$ channels influenced the $V_m$ and the bistable behaviour of mammalian skeletal muscle cells. The possible implication of this bistable behaviour in hypokalaemic periodic paralysis is discussed.

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Cells can have two stable steady-state membrane potentials ($V_m$) under identical conditions in media with lowered potassium concentration (Gadsby & Cranefield, 1977; Molgaard et al. 1980; Siegenbeek van Heukelom, 1991, 1994). This was found in a variety of myocardial and vascular cells, e.g. calf (Weidmann, 1956) and dog right ventricle trabecular cells (Gadsby & Cranefield, 1977), sheep Purkinje fibres (Carmeliet et al. 1987), human ventricular myocardial cells (McCullough et al. 1989), bovine aortic (Mehrke et al. 1991) and pulmonary artery endothelial cells (Voets et al. 1996). It was also observed in skeletal muscle fibres of frogs (Hodgkin & Horowicz, 1959; Nánás & Darñó, 1989), rats (Molgaard et al. 1980) and mice (Siegenbeek van Heukelom, 1991, 1994). Furthermore, it was found in a number of other cells, such as mouse macrophages (Gillin & Livengood, 1981) and rat (Sims & Dixon, 1989) and chicken osteoclasts (Ravesloot et al. 1989). This bistable behaviour, also called dichotomy or bimodality, is related to the negative slope conductance of the inward potassium rectifier ($K_R$; Gadsby & Cranefield, 1977). Some of the above cells were reported to switch from the hyperpolarized to the depolarized state (and vice versa) upon electrical stimulation in media with reduced potassium (Gadsby & Cranefield, 1977; Carmeliet et al. 1987).

We demonstrate here that this behaviour can also be evoked by reduction of potassium alone. If a cell becomes hyperpolarized when the potassium concentration ($K^+$) in the medium is lowered below 5.7 mM, then the cell membrane is regarded to be in a 'switched-on' state. However, if a cell becomes depolarized when $K^+$ is lowered below 5.7 mM, then the cell membrane is regarded as being in a 'switched-off' state and the $K^+$ at which such depolarization occurs is defined as the 'switch-off value' (Siegenbeek van Heukelom, 1994). When $K^+$ is reduced slowly, one finds that the switch-off value is smaller than the 'switch-on' value in one and the same cell; this phenomenon we call 'hysteresis'.

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Furthermore, this study shows that other processes influencing CI− movements across the plasma membrane, such as exposure to hypertonic media, inhibition of the Na+-K+-2Cl− cotransporter and blocking the conductive CI− channels, affect the electrical bistable behaviour of the muscle cells, as does Ba2+ block of K* and a change in temperature from 27 to 35°C. Some of these results have been presented in abstract form (Geuken Foppen et al. 1999).

METHODS

Preparation
Male and female white Swiss mice were housed and used in accordance with Dutch regulations concerning animal welfare. The mice were killed by cervical dislocation and the lumbar and diaphragmatic muscles were dissected and handled similarly.

Perfusion media and chemicals
The modified Krebs-Henseleit solution contained (mm): 117.5 NaCl, 5.7 KCl, 25.0 NaHCO3, 1.2 NaH2PO4, 2.5 CaCl2, 1.2 MgCl2, 4.44 glucose, saturated with humidified gas (95% O2−5% CO2); pH 7.35−7.45. K* concentrations were varied by equimolar replacement of NaCl by KCl or vice versa, thereby maintaining external CI− concentration constant throughout (150.6 mm). Isotonic solutions had an osmolality of 289 mmol kg−1 (± 6 mmol kg−1). All hypertonic solutions were made by addition of polyethylene glycol (PEG) with a molecular weight of 400 Da (PEG 400), large enough to be impermeant. The most common hypertonic solutions contained either 9.7 g PEG per litre (319 mmol kg−1) or 18.6 g PEG per litre (340 mmol kg−1). A more hypertonic medium contained 38 g PEG per litre (398 mmol kg−1), and a slightly less hypertonic medium (372 mmol kg−1) was made by diluting the most hypertonic medium with isotonic solution.

The osmotic values of all media used were expressed as osmolality, which was measured with a vapour-pressure osmometer (Wescor Model 5100C). Bumetanide and anthracene-9-carboxylic acid (9-AC) were added to the perfusion media in supramaximal concentrations (75 μM). 9-AC does not completely block the chloride conductance (Van Palade & Bacchi, 1977) and its potency depends on the extracellular chloride concentration (Astill et al. 1996). However, 75 μM 9-AC generated the maximal effect in our experimental conditions. All chemicals were of analytical grade. Salts were supplied by Janssen Chimica (Geel, Belgium), PEG 400 by Merck and all other chemicals by Sigma.

Measuring chamber
The measuring chamber, made of Sylgard 184 (Dow Corning, Midland, MI, USA), had a volume of approximately 0.1 ml and was continuously perfused (flow velocity 3 ml min−1). It was mounted on the object stage of an Olympus SZH microscope. Prewarmed solutions were transported to the chamber by means of a peristaltic pump (ISMATEC ISM 726, Ismatec SA, Zurich, Switzerland). Just before entering the chamber, the solution temperature was adjusted to 35.0 ± 0.5°C by passing it along a resistor heated by electrical current. Temperature was continuously measured with a K-type miniature thermometer (Keshley 871A, Cleveland, OH, USA) in the chamber. Turning the current off induced a drop in temperature to 27 ± 1°C. For fast temperature changes, a flush-through stainless-steel capillary was used to rapidly decrease (within 3 s) the temperature to 25 ± 0.5°C. The drop in temperature caused the bicarbonate-buffered solutions to acidify by less than 0.1 pH unit. The temperature dependence of ΔVm caused by solution changes was calculated using the equation ΔVm = (ΔVm/ΔT)ΔT, where ΔVm is the temperature (°C) and T1 > T2, and where ΔVm and ΔT are the membrane potential changes resulting from solution changes at temperatures T1 and T2 in the same cell.

Measurement procedure and the definition of Vm
Fine-tipped glass microelectrodes (containing 3 × KCl, tip resistance 25−80 MΩ) were used to measure Vm. They were all pulled on a Brown and Flaming puller (Sutter P87, Sutter, Novato, CA, USA). Criteria for recordings to be considered representative and definition of Vm have been described previously (Van Mil et al. 1997). The output of the microelectrode amplifier (WPI, M4-A) and the potential of the reference bath microelectrode were sampled (1 kHz) using LABView 3.1 (National Instruments, Austin, TX, USA). The data over 1 s were averaged and stored. We frequently used staircase protocols where we did not switch back to control solution but continued to change the solution composition (for example see Fig. 1). In the hysteresis measurements we also used staircase protocols, making very small steps by mixing different proportions of 2.85 and 0.76 mm K* containing solutions in steps of 10%.

![Figure 1. Effects of stepwise changes in K* on the membrane potential (Vm).](image-url)
Membrane state
The status of the cell membrane in the \( K^+ \) range between 0.76 and 3.8 mM was determined based on the value of \( V_m \) in relation to a threshold calculated by averaging an equal number of the most positive and the most negative \( V_m \) values for a specific \( K^+ \) at given osmolality. If the \( V_m \) was more negative than the threshold value, then the cell membrane was considered to be in the A-state and if \( V_m \) was more positive than the threshold value, then the membrane was considered to be in the B-state. This method for ascertaining the A- or B-state of the membrane is similar to the half-amplitude threshold technique routinely used to separate the open and the closed states of single channels (Colquhoun, 1994).

Statistics
Steady-state data, obtained at a particular \( K^+ \) and osmolality, are presented as means ± S.E.M. with the number of observations (n) in parentheses. The number of animals (N) is given when it differs from n. When mean values are compared, the significance was assessed using either Student's t test (n > 6) or the Mann-Whitney U test (n < 6). Unless indicated otherwise, P < 0.05 was assumed to indicate a significant difference. P was not calculated when n was less than 4. The correlation coefficient (r) is given when a curve was fitted to data.

RESULTS
Bistable behaviour is a cellular property
The staircase protocol for changing \( K^+ \) in Fig. 1 reveals that bistable behaviour is a property of an individual cell. First, \( K^+ \) was decreased stepwise from 5.7 mM to a value that made the cell depolarize to about −60 mV (when \( K^+ \) was made 2.05 mM the cell switched off). Then we increased \( K^+ \) to 2.32 mM again. The cell remained depolarized, whereas with the same concentration before the switch off it was hyperpolarized. Therefore, at the same concentration (here 2.32 mM) we found two different membrane potentials, one hyperpolarized (A-state) and one depolarized (B-state) with respect to the value in normal medium. The results in

![Figure 2. Hysteresis in isotonic and hypertonic solutions and in media containing bumetanide](image-url)

A, hysteresis in isotonic and hypertonic media in the same cell. \( V_m \) of the cell exhibited two stable values when \( K^+ \) was decreased and then increased in small steps. The hysteresis loop in isotonic media is shown in the left panel and the hysteresis loop at 340 mmol kg⁻¹ in the right panel. In both panels, the data with decreasing \( K^+ \) are connected by continuous lines and the data with increasing \( K^+ \) by dashed lines. The bar at the bottom of each panel indicates the range of \( K^+ \) where hysteresis occurred. B, hysteresis of \( V_m \) in isotonic media without and with bumetanide. In the left panel, for isotonic media without bumetanide, and in the right panel, for isotonic media containing bumetanide, \( K^+ \) was decreased and then increased in small steps. The data with decreasing \( K^+ \) are connected by continuous lines and the data with increasing \( K^+ \) by dashed lines. The last three data points of the increasing part of the staircase protocol in the isotonic media differ from the values expected on the basis of the results of decreasing part. However, when \( K^+ \) was 5.7 mM, \( V_m \) was −71.2 mV. This value is still sufficiently negative according to the criterion for a correct impalement (Van Mül et al. 1997). The bar at the bottom of each panel indicates the range of \( K^+ \) where hysteresis occurred.
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Fig. 1 were reproduced in seven other fibres (N = 7) with similar results.

Hysteresis exhibited by $V_m$ in response to small $K^+$ changes in isotonic, hypertonic and bumetanide-containing media

We also applied staircase protocols with smaller steps of approximately 0.2 mm $K^+$ (see Methods), and waited 4 min at each $K^+$ or 45 min when the switch value was crossed. After a 'control' staircase protocol in isotonic media was completed, a second staircase protocol was attempted in either hypertonic media (Fig. 2A) or isotonic media containing bumetanide (Fig. 2B). The completion of two successive full staircase protocols in one cell took about 3 h. In Fig. 2A the result of such a double staircase protocol is displayed. First, $K^+$ was reduced with an endpoint of 0.76 mm and the switch-off value was 1.6 mm. Then $K^+$ was increased, returning to 5.7 mm; the switch-on value was 2.64 mm. In every full experiment (n = 10) the switch-on value was higher than the switch-off value. After the control experiment, as shown in Fig. 2A, we conducted the experiment in a hypertonic solution (340 mmol kg$^{-1}$). Now we found a switch-off value of 2.85 mm and a switch-on value of 3.14 mm. The hysteresis loop was narrower than in isotonic media and shifted to a higher $K^+$.

Bumetanide shifted the hysteresis loop to lower $K^+$ values (Fig. 2B). The switch-off values decreased from a control value of 2.43 to 1.81 mm in bumetanide-containing media and the switch-on value decreased from 3.14 to 2.22 mm.

Similar recordings were obtained from three more cells in hypertonic media and two more cells in the presence of bumetanide. Also several other recordings (n = 4) where the cell membrane was damaged before completing the hysteresis loop further support this conclusion. Thus it appears that the Na$^+$–K$^+$–2Cl$^-$ cotransporter can facilitate the closure of $K_m$ at higher $K^+$.

The dependence of $V_m$ on $K^+$ and medium osmolality

The steady-state relationship between $V_m$ and $K^+$ was determined at three osmolalities (289, 319 and 340 mmol kg$^{-1}$; Fig. 3A). Because the previous results demonstrated bistable behaviour in media with lowered $K_m$, we separated our data into two groups: hyperpolarized cells in the A-state and depolarized cells in the B-state. All mean $V_m$ values of the A- and B-state with the same $K_m$ and osmolality differed significantly from one another (P < 0.01; see Fig. 3A). In hypertonic solutions, cells switched off at higher $K^+$. For cells in the B-state all slopes of the $V_m$-$K^+$ relation were similar and small. In the range between 5.7 and 22.8 mm (indicated by the horizontal bar in Fig. 3A) the slopes decreased with increasing osmolality from 50 mV per decade ($K^+$) at 289 mmol kg$^{-1}$ to 36 mV per decade at 319 mmol kg$^{-1}$ and 31 mV per decade at 340 mmol kg$^{-1}$.

Since Fig. 3A does not show how many cells hyperpolarized at lower $K^+$ values, we also plotted the fraction of hyperpolarized cells as a function of $K^+$ (Fig. 3B). This fraction diminished when the toxicity increased. Frequently, we found that in one muscle bundle some fibres hyperpolarized while others depolarized with the same $K^+$. On visual inspection no correlation could be found between fibre appearance and the status of the membrane.

$V_m$ can also be related to osmolality. At a $K^+$ of 5.7 mm a slope of 230 ± 10 mV mol$^{-1}$ kg (n = 157, N = 96) was found.
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with the three standard osmolalities. This dependence was also calculated at K_± values of 0.76 and 15 mM, and in a solution containing 5.7 mM K_± and 80 μM Ba^{2+}. These three solutions caused cells to depolarize to approximately −55 mV (B-state). In 0.76 mM K_±, the dependence was 48 ± 6 mV mol⁻¹ kg⁻¹ (n = 14, N = 10), in 15 mM K_±, it was 3.8 ± 12 mV mol⁻¹ kg⁻¹ (n = 14, N = 8) and in Ba^{2+}-containing media it was 50 ± 10 mV mol⁻¹ kg⁻¹ (n = 7, N = 5). All these values differ significantly (P < 0.001) from the sensitivity with a K_± of 5.7 mM. These results corroborate our previously published data with mannitol as the osmotic agent (Siegenbeek van Heukelom et al. 1994).

We also measured cell responses at 5.7 mM K_± in a wider range of hypertonicity than we reported earlier (Van Mil et al. 1997), but the preparation often deteriorated after exposure to osmolalities above 370 mmol kg⁻¹. This deterioration was observed in two ways. First, the depolarization was not always reversible and second, on microscopic inspection, the preparation frequently changed from transparent to nontransparent. Therefore, we have only a limited number of successful experiments 'above the (patho)physiological range' (approximately 350 mmol kg⁻¹; Hoffmann & Simonsen, 1989). V_m as a function of osmolality saturated at approximately −58 mV (Fig. 4).

Bistable behaviour at a K_± of 0.76 mM differs in various muscles

We set out to examine the variation in bistable behaviour among muscles. Therefore, we compared the fraction of hyperpolarizing cells when K_± was decreased from 5.7 to 0.76 mM in lumbricals, EDL, soleus and diaphragm muscle. At this concentration we found that in the lumbrical muscle 2.7% of the cells hyperpolarized (15 hyperpolarizations, n = 553, N = 250). In the EDL this was 70% (39 hyperpolarizations, n = 56, N = 30), in the soleus 32% (9 hyperpolarizations, n = 28, N = 21) and in diaphragm muscle 30% (3 hyperpolarizations, n = 10, N = 6). Compared to the lumbrical muscle, cells of all other muscles responded differently (P < 0.05 using the χ²-test). Even though the switch-off value measured using staircase protocols in lumbricals was between 1.3 and 2.5 mM (see Figs 2 and 3A), it can evidently be as low as a K_± of 0.76 mM, in rare cases.

Bumetanide, 9-AC and hypertonicity modulate the bistable behaviour

To demonstrate better that chloride transport influences the relation between V_m and K_±, we conducted experiments in which we applied bumetanide or 9-AC at various values of K_± and osmolality (see Table 1). At an osmolality of 340 mmol kg⁻¹ and 5.7 mM K_±, bumetanide had a larger effect than 9-AC (P < 0.05). Both agents had smaller effects when K_± was 15 mM or when cells were depolarized in low-K_± media. After addition of bumetanide, the application of 9-AC still induced a small hyperpolarization (ΔV_m = −1.5 ± 0.2 mV, n = 9, N = 8, P < 0.05) that was also evident in the hypertonic medium (340 mmol kg⁻¹; ΔV_m = −0.6 ± 0.2 mV, n = 6, N = 5, P < 0.05). This suggests an additional uphill Cl⁻ import (see Discussion).

Addition of bumetanide or 9-AC in the presence of Ba^{2+} (either in isotonic or hypertonic media) resulted in

![Figure 4. Increased osmolality causes cell depolarization in the presence of 5.7 mM K_±.](image)

The error bars represent s.e.m. at 289 mmol kg⁻¹; the error was smaller than the marker.

![Figure 5. Influence of the Na⁺-K⁺-2Cl⁻ cotransporter on bistable behaviour.](image)

A reduction of K_± from 5.7 to 2.85 mM induced a depolarization to the B-state (t = 10 min). Application of bumetanide caused the cell to hyperpolarize slightly, without switching the cell membrane to the A-state (t = 20 min). However, the cell remained in the A-state when bumetanide was added (at t = 49 min) before the same K_± reduction (t = 57 min). The experimental protocol is depicted above the recording, where K5.7 and K2.85 represent the K_± values of 5.7 and 2.85 mM, respectively, and Bum represents the application of bumetanide. Note that the two V_m values at t = 26 and t = 67 min are very different, though the composition of the extracellular solution is the same. In 4 cells, the average V_m values obtained thus in the A- or B-state with 2.85 mM K_± in the presence of bumetanide differed significantly: −87.4 ± 1.9 and −55.3 ± 1.3 mV, respectively.

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very small or nonsignificant hyperpolarizations; cells deteriorated quickly with the combined exposure to BaCl2, hypertonicity and bumetanide. Addition of bumetanide in normal medium caused a hyperpolarization not only in lumbrocolic muscle cells (−3.7 mV, Table 1), but also in cells from the diaphragm muscle (−2.9 ± 0.8 mV, n = 7, N = 6), soleus (−2.9 ± 1 mV, n = 4) and EDL (−2.8 ± 0.7 mV, n = 6, N = 5).

The effects of bumetanide and 9-AC on the bistable behaviour can also be demonstrated using the staircase protocol. Figure 5 demonstrates that cells which depolarized on decreasing KCl from 5.7 to 2.85 mM (at t = 10 min) could hyperpolarize in the presence of bumetanide (at t = 55 min), in response to the same KCl reduction. While the application of bumetanide during the depolarized state was ineffective (see Fig. 5 at t = 20 min) to switch on the cells depolarized from the A-state to the B-state (−55.9 mV, n = 2) due to the wash-out of bumetanide. The choice of 2.85 mM was made because even at this concentration cells could depolarize (see Fig. 3B) and this concentration is slightly above the estimated half-maximal KCl for the Na+-K+—2Cl− cotransporter (approximately 2 mM; Isenring & Forbush, 1997). When the medium was changed to a KCl of 0.76 mM, cells switched off even in the presence of bumetanide (n = 8, N = 7, see also Fig. 2B).

With the staircase protocol we found that addition of 9-AC, given before the KCl reduction, caused a cell to hyperpolarize (in Fig. 6 at t = 45 min), in contrast to the depolarization observed in the absence of 9-AC when KCl was reduced (at t = 5 min). All cells studied showed the same pattern; when 9-AC was given before the KCl reduction to 2.85 mM, they hyperpolarized (Vm = −86.8 ± 2.4 mV, n = 3), but not when 9-AC was given after the KCl reduction (Vm = −54.4 ± 3.3 mV, n = 3). Bistable behaviour was never observed when KCl was reduced to 0.76 mM (n = 7). Reducing KCl in the presence of 9-AC sometimes induced oscillations of Vm, most probably because of the removal of the damping effect exerted by G0. In Fig. 6 such oscillations are shown, when KCl was switched from 2.85 to 5.7 mM (t = 23–28 min). We also sometimes found spikes.
that we identified as myotonic discharges because we frequently lost the cell at that moment. In Fig. 6 at $t = 60$ min spikes started to manifest themselves in the presence of 9-AC and the G_b blocker was removed before the cell was damaged.

**Effects of Ba$^{2+}$ on electrical bistable behaviour**

Since Ba$^{2+}$ blocks the current through K_m we also studied the effect of washing out Ba$^{2+}$ from the preparation. Figure 7 shows that in normal medium the cell repolarized from the B-state to the A-state when Ba$^{2+}$ (80 μM) was washed out. This was not the case when K_m was 2.85 mM ($n = 5$). The depolarization after washing Ba$^{2+}$ out in normal medium with 5.7 mM K_m was not influenced by 9-AC ($n = 5$) or hypertonicity ($n = 5, N = 4$).

**The effects of temperature**

Data on the effects of hypertonicity and the introduction of bumetanide or 9-AC at 35 and 27°C in hypertonic media with a K_m of 5.7 mM are compared in Table 2. The experiments were carried out at 340 mmol kg$^{-1}$ to increase the responses to bumetanide and 9-AC (see Table 1). The depolarizations induced by hypertonicity and the hyperpolarizations induced by bumetanide were significantly smaller at 27°C compared to those at 35°C. Remarkably, the hyperpolarizations due to 9-AC in hypertonic media were increased at lower temperature (Table 2). The cell responses to 80 μM Ba$^{2+}$ at 35 and 27°C did not differ significantly ($P > 0.05, n = 6, N = 5$).

The effects of temperature changes are illustrated in Fig. 8, which shows one of seven similar recordings. Temperature was rapidly changed (approximately 3 s) from 35 to 27°C and vice versa. When cells were cooled in a medium with a K_m of 2.85 mM, they switched off ($\Delta V_m = 37 \pm 3$ mV, $n = 6$). However, on one occasion a cell did not switch off; we found $V_m = -88.1$ mV (35°C), decreasing to $-86.6$ mV (27°C) and then increasing to $-89.9$ mV (35°C). When a steady state was reached at 27°C ($V_m = -48 \pm 3$ mV, $n = 6$), the temperature was increased again to 35°C. Two cells hyperpolarized completely to approximately $-82$ mV and four did not ($\Delta V_m = -4 \pm 4$ mV). Together with the results of other recordings, less complete than the one in Fig. 8, in ten depolarized cells at 27°C we found a switch to the A-state on increasing temperature ($\Delta V_m = -34 \pm 1$ mV) on six occasions but not on four others ($\Delta V_m = -5 \pm 3$ mV). This bistable behaviour was never found when K_m was 5.7 mM ($n = 10$).

Figure 8 (from approximately 70 min onwards) also illustrates, like Fig. 5, that the addition of bumetanide can aid a cell to maintain the A-state on reduction of K_m. This was found in all cells we tested with this protocol ($\Delta V_m = 1 \pm 1.7$ mV, $n = 3$).

**DISCUSSION**

Bistable behaviour expressed as two stable membrane potentials has been observed for different types of cells in media with reduced potassium concentration with respect to control media and it is generally agreed that this behaviour is caused by the properties of the K_m (see...
How does Cl\textsuperscript- transport influence the bistable behaviour of muscle cells?

As mentioned earlier, electrical bistable behaviour of the cell is caused by the properties of \( K_m \), which is the dominant element of the potassium permeability (\( P_K \)), as described by the following equation (Siegenbeek van Heukelom, 1994):

\[
P_K = P_r + P_{K_{\text{max}}} \left[ \sqrt{K_r} \left[ 1 + \exp \left( \frac{(V_m - E_K)/V_C}{\sqrt{K_r}} \right) \right] \right]^{-1},
\]

where \( P_r \) is a residual, \( K_m \)-independent potassium permeability, which makes \( V_m \) become about 50 mV when \( K_m \) is closed. This is the reversal potential for the equimolar exchange of potassium and sodium. \( P_{K_{\text{max}}} \) specifies the maximal steady-state permeability of \( K_m \). It is divided by the square root of \( K_r \) and the Boltzmann partition function that describes the kinetic behaviour of \( K_m \) (Hille, 1992). \( V_C \) gives the steepness of the voltage dependence. This expression fits the experimental data of Standen & Stanfield (1978). Owing to the Boltzmann distribution, \( K_m \) already opens at \( V_m \) values slightly less negative than the potassium equilibrium potential, \( E_K \).

Without chloride transport, the equations (Siegenbeek van Heukelom, 1994) describing the balance of cations and the activity of the \( Na^+-K^+ \)-pump suffice to explain the bistable behaviour and hysteresis. Experiments with bumetanide exemplify this situation.

The \( Na^+-K^+ \)-2Cl\textsuperscript- cotransporter and large chloride conductance present a depolarizing influence on the membrane potential. Uphill import of chloride through the \( Na^+-K^+ \)-2Cl\textsuperscript- cotransporter will lead to accumulation of chloride in the cell and the equilibrium potential of chloride (\( E_C \)) will be positive with respect to \( V_m \). Because of the large chloride conductance, the difference \( V_m - E_C \) is small. Nevertheless, the depolarizing influence of \( E_C \) on \( V_m \) leads to an increase of \( V_m - E_K \) in the denominator of the Boltzmann equation (Van Mil, 1998). As the exponential in the denominator increases, \( P_r \) decreases, because the \( K_m \) slider slides down its permeability—voltage relation. This process can force \( K_m \) to close regeneratively, and \( V_m \) attains a new steady-state value of approximately 50 mV, where \( P_K = P_r \).

The above-mentioned mechanism is enhanced in hypertonic solutions because of two collaborating effects. First, cell shrinkage will occur, though it must be so small that we could not detect it visually. This shrinkage by itself concentrates potassium and chloride in the cell and leads to an increase in \( V_m - E_K \), because \( E_K \) becomes more negative and \( E_C \) more positive with respect to \( V_m \). Second, stimulation of the \( Na^+-K^+ \)-2Cl\textsuperscript- cotransporter by hypertonicity enhances the chloride accumulation in the cell. \( E_C \) and \( V_m \) will become less negative. The coupled \( K^+ \) import will simultaneously make \( E_K \) more negative. Therefore, hypertonicity will promote the regenerative closure of \( K_m \), as mentioned above.

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**Figure 8. Temperature reduction can induce the switch off**

Cells normally switched off on temperature reduction from 35 to 27°C with a \( K_r \) of 2.85 mV (at \( t = 12 \) min in this figure; see protocol indications above the recording) and could not repolarize on warming up again (at \( t = 45 \) min). Thus, at 35°C and with a \( K_r \) of 2.85 mV, owing to its bistable behaviour the cell had two stable values of \( V_m \), at \( t = 10 \) min (\( V_m = 84 \) mV) and at \( t = 45 \) min (\( V_m = 55 \) mV). The change in temperature was achieved in approximately 3 s. As shown in Fig. 5, the switch off could be prevented by inhibition of the \( Na^+-K^+ \)-2Cl\textsuperscript- cotransporter. Six similar recordings (\( N = 6 \)) of the first part (until approximately 60 min) and three (\( N = 3 \)) of the last part were obtained.
A full description can only be given by solving all equations involved simultaneously (Geukes Poppen et al. 2001). These equations do not explain why the $V_m$ of cells with closed $K_a$ do not demonstrate strong dependence on osmolality in all cases. However, small variations were observed sometimes (see Fig. 5 at $t = 20$ min, when addition of bumetanide evokers a small negative $\Delta V_m$). Two possible processes might be the reason. First, due to the reduction of $V_m$ the driving force for uphill transport, the sodium gradient, is completely reduced. Second, the closure of $K_a$ impeds the efflux of potassium and influences the intracellular concentrations of sodium and potassium unfavorably for uphill transport of chloride (see also the next paragraph). Only the measurement of intracellular cation concentrations can provide data that document whether the electrical or chemical component of the sodium gradient is most important.

**Effects of 9-AC and bumetanide compared**

Bumetanide and 9-AC both reduced the chloride efflux as a positive current into the cell, bumetanide by reducing the driving force and 9-AC by reducing $G_C$. With both agents the cell remained more easily in the A-state. When $K_a$ was 5.7 ms their effect appeared to be optimal. When cells exhibited bistable behaviour, application of bumetanide or 9-AC did not induce cells in the B-state to switch to the A-state. In the B-state $V_m - E_K$ is so large that, most probably, the Na"--K"--2Cl" complex pump is not powerful enough to repolarize the cell and reopen the $K_a$. When $K_a$ was 15 ms the influence of hypotonicity, bumetanide or 9-AC were small because the potassium gradient across the membrane had been reduced considerably and $V_m$ approached $E_K$.

The effect of 9-AC on the membrane potential consists of two elements, the inhibition of $G_O$ (with a hyperpolarizing effect) and the subsequent augmented chloride accumulation (with a depolarizing effect). In isotonic medium, the hyperpolarizations due to 9-AC and bumetanide compare well (~3 vs. 3.7 mV). However, in hypertonic medium (340 mmol kg"^"^-1) the mean $\Delta V_m$ due to 9-AC is -5.2 mV, and due to bumetanide -12.9 mV, most probably because 9-AC is not a complete blocker of $G_O$ (Palade & Barchi, 1977).

The fact that 9-AC induced a $\Delta V_m = -1$ mV in both iso- and hypertonic media containing bumetanide, might indicate an additional bumetanide-insensitive Cl" entry (Davis, 1996; Chipperfield et al. 1997). A second explanation may be that this residual potential change is caused by a small HCO$_3$" permeability ($P_{HCO_3}$) of the CIC-1 channels. Rychkov et al. (1998) mentioned that $P_{HCO_3}/P_C$ of CIC-1 is 0.027. The equilibrium potential of protons is between -10 and -30 mV (Aickin & Thomas, 1977), which is equal to the equilibrium potential of HCO$_3$" ($E_{HCO_3}$) in bicarbonate buffers with constant $P_{CO_2}$. These values for $P_{HCO_3}$ and $E_{HCO_3}$ are sufficient to account for a shift in reversal potential for CIC-1 of approximately 1 mV. The alternative explanation, that bumetanide does not inhibit all the active Na"--K"--2Cl" cotransporters, seems unlikely because the concentration we used appears to be supramaximal (Van Mil et al. 1997).

**Temperature dependence of Cl" transport**

At 27°C, $\Delta V_m$ induced by hyperpolarization and by bumetanide were both about half the values observed at 35°C. This suggests that the activity of the Na"--K"--2Cl" cotransporter is highly temperature dependent, in accordance with the finding of Lylet et al. (1998). Therefore, at 35°C cells might accumulate more chloride than the 1.4 ms at room temperature reported by Aickin et al. (1989). If $G_O$ is temperature insensitive, as reported by Palade & Barchi (1977) in rat diaphragm muscle (25-40°C), it is understandable why the hyperpolarization induced by 9-AC depended inversely on temperature. At 27°C and 340 mmol kg"^-1 the effects of bumetanide and 9-AC were equal (see Table 2), because the two elements of the effect of 9-AC, hyperpolarization due to the reduction of $G_O$ and depolarization due to accumulation of chloride, made them comparable. At 35°C, however, the Na"--K"--2Cl" cotransporter was more vigorous and achieved more chloride accumulation with blocked $G_O$.

**Discrepancies in $\Delta V_m$ in skeletal muscle reported in the literature**

Our data can explain apparent discrepancies in $\Delta V_m$ found in the literature. The inverse temperature dependence of hyperpolarization induced by 9-AC can explain why Aickin et al. (1989) reported a value of 10-15 mV at room temperature, whereas we found 3 mV. Donaldson & Leader (1984) reported that in media of 290 mmol kg"^-1 there was no chloride accumulation in the EDL of the mouse, whereas Dulhunty (1978), using 335 mmol kg"^-1, concluded that chloride is accumulated actively in the same preparation. Additionally, different $\Delta V_m/\Delta K_a$ values were reported for rat soleus muscle and for mouse EDL. For soleus Matgaard et al. (1980) reported a $\Delta V_m/\Delta K_a$ of 52.5 mV per decade at 288 mmol kg"^-1, while Chua & Dulhunty (1988) found a sensitivity of 36 mV per decade at 335 mmol kg"^-1. As for the EDL of mice, Siemenbeek van Heukelom (1991) measured at 289 mmol kg"^-1 55 mV per decade, whereas Dulhunty (1980) found 36 mV per decade at 335 mmol kg"^-1. In the present study, in mouse lumbrical muscle fibre, we found a decline of $\Delta V_m/\Delta K_a$ from 50 mV per decade in 289 mmol kg"^-1 to 31 mV per decade in 340 mmol kg"^-1. From our results we conclude that, taking into account chloride transport and its dependence on tonicity and temperature, it is possible to explain the discrepancies in these data in the literature.

**Physiological role**

Though intracellular Mg" or polyamines appear to be the rectifying agents of $K_a$ (Nichols & Lopatin, 1997), we only
studied the influence of monovalent ion transport systems on $V_m$ as a parameter of total cell behaviour.

Gallant (1983) concluded that treatment of mammalian skeletal muscle cells with $Ba^{2+}$-induced $Na^+$ current a number of effects similar to those observed during hypokalaemic periodic paralysis. $Ba^{2+}$ application can prevent excessive loss of potassium but jeopardizes contractile force development, as is the case after exercise or during an episode of hypokalaemic periodic paralysis (Claussen & Overgaard, 2000). The reduced potassium permeability mentioned by Gallant (1983) can be interpreted as the closure of the $K_a$-channel with our earlier observations (Siegenbeek van Heukelom, 1991, 1994; Van Mil et al. 1995), we conclude that our present observations might provide additional insight into hypokalaemic periodic paralysis (Barcl, 1994).

At lower temperatures, as is the case in the body extremities, the closure of $K_a$ will be most marked. Individual cells with closed $K_a$ will accumulate potassium, thus reducing the extracellular potassium concentration in restricted spaces. In turn, this reduction in extracellular potassium can cause the closure of $K_a$ in the neighbouring cells and consequently these cells may also depolarize, thus increasing the number of depolarized cells. Although this bistable behaviour of the cells is mainly caused by the highly nonlinear character of $K_a$, it is nevertheless susceptible to influences of other transport mechanisms, as shown in this study.

REFERENCES


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