



## UvA-DARE (Digital Academic Repository)

### The influence of bumetanide on the membrane potential of mouse skeletal muscle cells in isotonic and hypertonic media

van Mil, H.G.J.; Geukes Foppen, R.J.; Siegenbeek van Heukelom, J.

**DOI**

[10.1038/sj.bjp.0700887](https://doi.org/10.1038/sj.bjp.0700887)

**Publication date**

1997

**Published in**

British Journal of Pharmacology

[Link to publication](#)

**Citation for published version (APA):**

van Mil, H. G. J., Geukes Foppen, R. J., & Siegenbeek van Heukelom, J. (1997). The influence of bumetanide on the membrane potential of mouse skeletal muscle cells in isotonic and hypertonic media. *British Journal of Pharmacology*, *120*, 39-44.  
<https://doi.org/10.1038/sj.bjp.0700887>

**General rights**

It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

**Disclaimer/Complaints regulations**

If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: <https://uba.uva.nl/en/contact>, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.



# The influence of bumetanide on the membrane potential of mouse skeletal muscle cells in isotonic and hypertonic media

<sup>1</sup>H.G.J. van Mil, R.J. Geukes Foppen and J. Siegenbeek van Heukelom

Graduate School for Neurosciences Amsterdam, Institute of Neurobiology; Group Cell Biophysics, University of Amsterdam, Kruislaan 320, 1098 SM Amsterdam, The Netherlands

- 1 Increasing the medium osmolality, with a non-ionic osmoticant, from control (289 mOsm) to 319 mOsm or 344 mOsm in the lumbrical muscle cell of the mouse, resulted in a depolarization of the membrane potential ( $V_m$ ) of 5.9 mV and 10.9 mV, respectively.
- 2 In control medium, the blockers of chloride related cotransport bumetanide and furosemide, induced a hyperpolarization of  $-3.6$  and  $-3.0$  mV and prevented the depolarization due to hypertonicity. When bumetanide was added in hypertonic media  $V_m$  fully repolarized to control values.
- 3 In a medium of 266 mOsm, the hyperpolarization by bumetanide was absent.
- 4 At 344 mOsm the half-maximal effective concentration ( $IC_{50}$ ) was  $0.5 \mu M$  for bumetanide and  $21 \mu M$  for furosemide.
- 5 In solutions containing 1.25 mM sodium the depolarization by hypertonicity was reduced to 2.3 mV.
- 6 Reducing chloride permeability, by anthracene 9 carboxylic acid (9-AC) in 289 mOsm, induced a small but significant hyperpolarization of  $-2.6$  mV. Increasing medium osmolality to 344 mOsm enlarged this hyperpolarization significantly to  $-7.6$  mV.
- 7 In a solution of 344 mOsm containing  $100 \mu M$  ouabain, the bumetanide-induced hyperpolarization of  $V_m$  was absent.
- 8 The results indicate that a Na-K-2Cl cotransporter is present in mouse lumbrical muscle fibre and that its contribution to  $V_m$  is dependent on medium osmolality.

**Keywords:** Bumetanide; furosemide; osmoregulation; Na-K-2Cl cotransporter; membrane potential; ouabain, Na/K-pump, skeletal muscle

## Introduction

The membrane potential ( $V_m$ ), of mouse isolated lumbrical muscle fibre, is sensitive to the osmotic value of the superfusion medium. Hypertonicity induced a depolarization that was not transient but maintained (Siegenbeek van Heukelom *et al.*, 1994). It was shown that in the physiological range the  $V_m$  of these fibres is more responsive to the medium osmolality than to medium potassium concentration  $K^+$ .

Blinks (1965) and Chinet & Giovannini (1989) showed that skeletal muscle fibres shrink to a new steady state volume during hypertonicity. If one assumes that the chloride distribution is in equilibrium, cell shrinkage will lead to a hyperpolarization, due to the increase of the intracellular cation concentration and the higher permeability for potassium compared to sodium. Indeed, in frog toe muscle fibre Gordon & Godt (1970) found a hyperpolarization when medium osmolality was increased from 235 mOsm to 460 mOsm.

Aickin *et al.*, (1989) demonstrated, under normal physiological conditions, in rat lumbrical muscle cells a furosemide-sensitive Na-K-2Cl cotransporter which maintains a chloride concentration above equilibrium. Blocking the chloride permeability ( $P_{Cl}$ ) with anthracene-9-carboxylic acid (9-AC) resulted in a small hyperpolarization that was accompanied by an increase in intracellular chloride activity. In mammalian skeletal muscle fibres  $P_{Cl}$  is 3 to 20 times larger than the potassium permeability ( $P_K$ ), which implies that  $V_m$  follows the chloride equilibrium potential ( $E_{Cl}$ ) closer than the potassium equilibrium potential. Any disequilibrium of the intracellular chloride concentration will become manifested in  $V_m$  (Aickin, 1990). Dulhunty (1978), executing chloride substitution experiments, observed a considerably higher contribution of the chloride distribution to  $V_m$  than Aickin *et al.* (1989). The

control solutions used by Dulhunty (1978) were hypertonic (340 mOsm) compared to the solutions used by Aickin *et al.* (1989, approx. 295 mOsm). This supports the idea that in muscle cells chloride is accumulated above equilibrium under resting physiological conditions and that this accumulation is enhanced by hypertonicity. In rat soleus muscle fibres the inhibition of chloride related cotransport with bumetanide decreased the energy dissipation and sodium influx in control conditions (Chinet, 1993). Increased sodium import and energy dissipation occurred when the hypertonicity of the media was increased. These increments were blocked by bumetanide and amiloride indicating a contribution of a Na-Cl cotransporter and a Na-H exchanger (Chinet, 1993). This suggests that the accumulation is an energy requiring process.

To investigate the role of a chloride related cotransporter in the response of skeletal muscle cells to hypertonicity, we studied the influence on  $V_m$  of bumetanide as an inhibitor of Cl related cotransport (Weiner & Mudge, 1990), 9-AC as a blocker of  $P_{Cl}$ , and ouabain as an inhibitor of the Na/K-pump. Some of these data have been presented in a preliminary form (van Mil *et al.*, 1995a).

## Methods

### Preparation and experimental procedures

White Swiss mice (age 8 to 18 weeks, weighing 20–40 g, of either sex) were killed by cervical dislocation. The lumbrical muscle fibres were removed from a hind foot. One bundle was cut free and cleaned from connective tissue as described previously (van Mil *et al.*, 1995b). Superficial cells were impaled with fine-tipped microelectrodes (3 M KCl: 25–80 M $\Omega$ ). Impalements were considered successful and measurements were continued if  $V_m$  was steady and more negative than  $-70$  mV

<sup>1</sup> Author for correspondence

in control medium. Most data are derived from paired measurements (see statistics). When an active substance was washed out ('washout') the new steady state  $V_m$  value should differ by less than 3 mV from the value before the application of the substance. Similar criteria apply when substances are used in succession. Measurements were considered successful if  $V_m$  also returned to the original value ( $\pm 4$  mV) after the solution was switched back to control medium. More details have been described previously (Siegenbeek van Heukelom, 1991).

#### Measuring chamber, definition of $V_m$ and chemicals

The measuring chamber, made of Sylgard 184 (Dow Corning, Mich. 48640, U.S.A.), had a volume of approximately 0.1 ml and was continuously perfused ( $3 \text{ ml min}^{-1}$ ) at  $35 \pm 1^\circ\text{C}$ . The Krebs-Henseleit solution (referred to as control) contained (in mM): NaCl 117.5, KCl 5.7,  $\text{NaHCO}_3$  25.0,  $\text{NaH}_2\text{PO}_4$  1.2,  $\text{CaCl}_2$  2.5,  $\text{MgSO}_4$  1.2 and glucose 5.6, saturated with humidified gas: 95%  $\text{O}_2$  + 5%  $\text{CO}_2$ ; pH = 7.35–7.45. A 1.25 mM  $\text{Na}^+$  medium was made by replacing  $\text{NaHCO}_3$  with Cholinebicarbonate ( $\text{CholHCO}_3$ ) and all NaCl with N-methyl-D-glucamine-chloride salt (NMDG-C1) made by titrating NMDG to pH 7.2 with HCl. The ionic strength of the solutions was approximately constant.

All hypertonic solutions were made by adding polyethyleneglycol-400 (PEG with  $\text{MW} \approx 400$ ). The osmotic value of all media used was measured with the Wescor Vapour-pressure osmometer Model 5100C. This instrument determines the osmolality ( $\text{mol kg}^{-1}$ ) of the solutions and all osmotic values are expressed accordingly. The osmolality of the control solutions was  $289 \pm 1$  mOsm; the osmolality of all hypertonic solutions containing  $9.7 \text{ gl}^{-1}$  PEG was  $319 \pm 1$  mOsm and with  $18.6 \text{ gl}^{-1}$  PEG it was  $344 \pm 1$  mOsm. In a number of measurements the osmolality was reduced to 266 mOsm by lowering the NaCl concentration to 105 mM. The hypertonicities used were within the (patho) physiological range: 225 to 350 mOsm (Hoffman & Simonsen, 1989).

All chemicals were analytically pure; salts were from Janssen Chimica, ouabain (g-Strophanthidin krist. reinst) and polyethyleneglycol were from Merck and all other chemicals from Sigma. Bumetanide, furosemide and 9-AC were dissolved in methanol. These stock solutions were diluted 1000 times to obtain the concentration that was needed for the experiment.

#### Statistics

Results are presented in the text as mean  $\pm$  s.e.mean with the number of measurements from different cells presented between parentheses. We give the results of the averaged  $V_m$  value to one decimal place; the s.e.mean reflects the error due to stochastic influences. Influences of electrode junction potentials were minimized by defining  $V_m$  as the potential difference between the microelectrode in the cell and an identical microelectrode outside the cell. Nevertheless, we are aware of the systematic error due to the differences in the liquid junction potentials of the electrodes in the medium and in the cytosol, that Barry & Lynch (1991, see also Barry & Diamond, 1970) estimated to be of the order of 2 mV. We did not introduce corrections for this because it is not clear how these errors change due to hypertonicity. Changes in  $V_m$  ( $\Delta V_m$ ) are always expressed as the difference in the same cell between the steady state values before and after the change in solution. Groups of measurements were compared to one another by either two tailed Student's *t* test (when numbers of measurements were large and normally distributed) or Mann-Whitney (when numbers were smaller than 6 or not normally distributed). When a curve was fitted to the data, the quality of the fit was expressed by the correlation coefficient, *r*.

Differences in mean values were considered statistical not significant (NS) when  $P \geq 0.05$  and significant when  $P < 0.05$ . When numbers of measurements were less than 5 no com-

parison was made (-). As nearly all data and conclusions are related to changes observed in the cell, it is unlikely that the differences in liquid junction potentials severely change the significance assigned by us to the observed differences.

## Results

#### Depolarization of $V_m$ by increasing hypertonicity

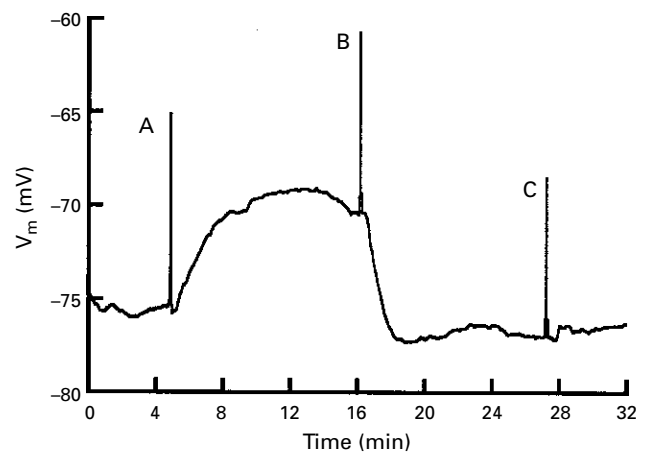
When the osmolality was increased from  $289 \pm 1$  mOsm (control) to  $319 \pm 1$  ( $n=46$ ) or  $344 \pm 1$  mOsm ( $n=50$ ), a depolarization was observed of  $5.9 \pm 0.4$  mV ( $n=46$ ) (see Figure 1) or  $10.9 \pm 0.5$  mV ( $n=50$ ) respectively. The  $V_m$  in 319 mOsm was significantly more negative than in 344 mOsm ( $P < 0.001$ ). This depolarization was reversible: return to iso-osmotic solutions hyperpolarized  $V_m$  with respectively  $-5.1 \pm 0.8$  mV ( $n=20$ ) or  $-9.8 \pm 1.1$  mV ( $n=17$ ) ( $P < 0.05$ , paired data). The sensitivity of  $V_m$  for medium osmolality ( $\Delta V_m/\Delta \text{Osm} \approx 0.2 \text{ V/Osm}$ ) was the same as measured previously with mannitol (MW 182) (Siegenbeek van Heukelom *et al.*, 1994).

We also performed a few experiments at higher concentrations (up to 465 mOsm). The results were qualitatively the same, though they appeared to show saturation. The  $V_m$  in 465 mOsm medium ( $-52.1 \pm 0.5$  mV ( $n=5$ )) was comparable to the maximally depolarized  $V_m$  in 344 mOsm medium.

#### The influence of bumetanide on $V_m$ in control and hypertonic media

When a supramaximal concentration ( $75 \mu\text{M}$ ) of bumetanide was applied to the muscle cells in control (289 mOsm) solution a reversible hyperpolarization,  $\Delta V_m = -3.6$  mV, was observed (see Table 1). The same observation was made when using furosemide ( $100 \mu\text{M}$ ):  $\Delta V_m = -3.0$  mV. Pretreatment with bumetanide prevented the depolarization by 344 mOsm (see Table 1;  $P > 0.05$ ). The  $V_m$  in 344 mOsm with bumetanide was stable for up to 90 min. We never observed an ongoing change in  $V_m$  under these conditions.

The hyperpolarization induced by bumetanide increased with increasing osmolality. Compared to 289 mOsm bumetanide ( $75 \mu\text{M}$ ) induced a significantly greater hyperpolarization in solutions of 319 mOsm or 344 mOsm of  $-7.1$  mV ( $P < 0.05$ ) and  $-12.9$  mV ( $P < 0.001$ ), respectively (see Figures 1 and 2). The  $V_m$  values attained in hypertonic media with bumetanide were more negative than in 289 mOsm without



**Figure 1** Typical recording demonstrating the  $V_m$  response to hyperosmotic stress (319 mOsm) and bumetanide. (A) 25 mOsm PEG was added which lead to a depolarization followed by a partial repolarization. (B) Bumetanide was added and restored  $V_m$  completely with a small overcompensation. (C) Hypertonicity and bumetanide were washed out simultaneously resulting in a small depolarization.

**Table 1** Response of  $V_m$  to bumetanide or furosemide under different conditions

Initial conditions	$V_m$ (mV)	Experimental challenge	$\Delta V_m$ (mV)	n	P
289 mOsm	$-74.8 \pm 0.9$	Bumet	$-3.6 \pm 0.8$	13	**
289 mOsm	$-77.5 \pm 2.3$	Furo	$-3.0 \pm 1.0$	5	*
289 mOsm + Bumet	$-77.4 \pm 1.2$	344 mOsm + Bumet	$1.1 \pm 0.6$	6	NS
319 mOsm	$-69.5 \pm 1.8$	Bumet	$-7.1 \pm 1.4$	7	**
319 mOsm	$-68.1 \pm 3.9$	Furo	$-5.7 \pm 2.2$	6	*
344 mOsm	$-62.5 \pm 1.5$	Bumet	$-12.9 \pm 1.3$	20	**
344 mOsm	$-66.4 \pm 3.6$	Furo	$-9.6 \pm 1.2$	4	–
344 mOsm + Bumet	$-76.3 \pm 0.8$	289 mOsm + Bumet	$0.3 \pm 0.6$	5	NS

Bumetanide (Bumet,  $75 \mu\text{M}$ ) and furosemide (Furo,  $200 \mu\text{M}$ ) were added in control solution of 289 mOsm (rows 1 and 2); in medium with approximately 319 mOsm (rows 4 and 5) or 344 mOsm (rows 6 and 7). Increasing osmolality to 344 mOsm in the presence of bumetanide (row 3) did not change  $\Delta V_m$ . Row 8 shows complete suppression of hypertonic depolarizations by bumetanide including the overshoot. Data shown as means  $\pm$  s.e.mean. Significance ( $P$ ) is given for  $\Delta V_m$  compared to zero: \*\* $P < 0.01$ ; \* $P < 0.05$ ; NS not significant  $P > 0.05$ .

bumetanide: we observed an overshoot of  $-1.5 \pm 0.5$  mV ( $n = 7$ ) in 319 mOsm and  $-2.1 \pm 0.9$  mV ( $n = 16$ ) in 344 mOsm. Figure 1 shows a typical example of such an overshoot of  $V_m$ . This overshoot effect was not observed with furosemide ( $100 \mu\text{M}$ ).

The same influence of bumetanide was found in 465 mOsm: it also reversed the depolarization.

Chinet (1993) found a contribution of the Na-H exchanger in the osmoregulation in rat soleus muscle fibres. Amiloride, in concentrations of 1 mM, inhibits the Na-H exchanger. We found no significant effect of 1 mM amiloride on  $V_m$  in control or hypertonic solutions.

#### Bumetanide effect in medium of 266 mOsm

A plot of the response of bumetanide as function of the osmolality suggested that its effect might be zero when the osmolality was reduced to 266 mOsm (see Figure 2). When osmolality was reduced to 266 mOsm by decreasing NaCl to 105 mM,  $V_m$  did not significantly change. In this medium we added bumetanide and the response did not differ significantly from zero:  $\Delta V_m = -0.3 \pm 0.2$  mV ( $n = 6$ ). To ascertain that the reductions of  $\text{Na}^+$  and  $\text{Cl}^-$  were not the origin of this observation we added 20 mM PEG to this medium restoring the medium osmolality to 289 mOsm. In this medium bumetanide induced a  $-3.4 \pm 0.7$  mV ( $n = 5$ ) hyperpolarization that did not differ significantly from those obtained in the control medium (see Table 1).

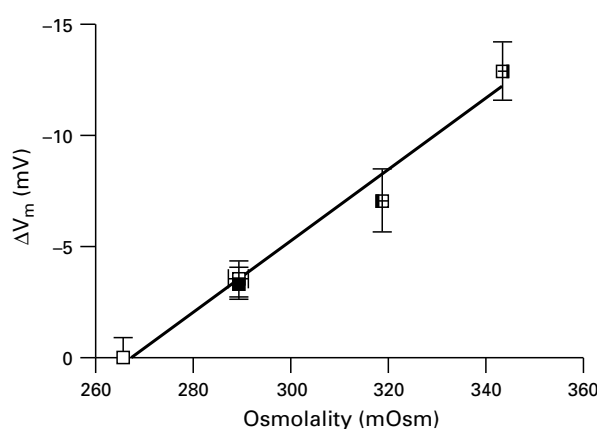
#### Dose-response data of bumetanide and furosemide

Bumetanide and furosemide are known to block a variety of chloride related cotransporters (Haas, 1994). Dose-response curves for bumetanide and furosemide were determined in media with 344 mOsm (Figure 3). We normalized all data by expressing the maximal value in one cell as 100% and the other  $V_m$  values in the same cell as a fraction of this maximal value.

The cells showed a significantly greater sensitivity ( $P < 0.001$ ) for bumetanide ( $\text{IC}_{50} = 0.5 \pm 0.02 \mu\text{M}$ ) than for furosemide ( $\text{IC}_{50} = 21 \pm 9 \mu\text{M}$ ). In a few experiments we verified that bumetanide in addition to furosemide did not influence  $V_m$ ; nor did furosemide in addition to bumetanide. Furosemide unlike bumetanide appeared to have a deteriorating effect on the muscle cells. When the muscle cells were exposed to furosemide in concentrations higher than  $100 \mu\text{M}$  and for longer than 10 min, the impaled cell was lost and it became increasingly difficult to impale ( $V_m < -70$  mV in control solution) a new cell successfully in the same preparation. For this reason we used bumetanide in these experiments.

#### Hyperosmotic stress in 1.25 mM $\text{Na}^+$ solutions

Bumetanide and furosemide are known to block a number of chloride-cotransporting systems. To find out whether sodium



**Figure 2** Hyperpolarizations induced by bumetanide at different medium osmolalities. When the data (open symbols) were fitted with a straight line,  $\Delta V_m = 44.5 - 0.165 \times \text{Osm}$  ( $r = 0.99$ ) and  $\Delta V_m = 0$  at 269 mOsm. The two points at 289 mOsm represent the hyperpolarization in control solution (open symbol) and the value when NaCl was lowered but the osmolality was restored by addition of PEG (solid symbol).

is an obligatory cotransported ion we conducted experiments in solutions containing 1.25 mM  $\text{Na}^+$ .

Probably due to the method of composing this 1.25 mM solution, it had a higher osmolality than the control solution:  $327 \pm 4$  mOsm ( $n = 6$ ). However, no significant change of  $V_m$  was noticed ( $\Delta V_m = 0.6 \pm 1.4$  mV ( $n = 6$ ),  $P > 0.05$ ) when  $\text{Na}^+$  was changed to 1.25 M, before as well as after such a change in solution. After exposure to 1.25 mM  $\text{Na}^+$  the cell was still capable of responding normally to further experiments, as a subsequent increase to 368 mOsm with 50 mM PEG led to a small depolarization of  $2.3 \pm 0.3$  mV ( $n = 6$ ) that was significantly different from zero ( $P < 0.01$ ). However this depolarization was significantly smaller than  $\Delta V_m$  due to the application of the same osmotic shock in control solution ( $P < 0.001$ ).

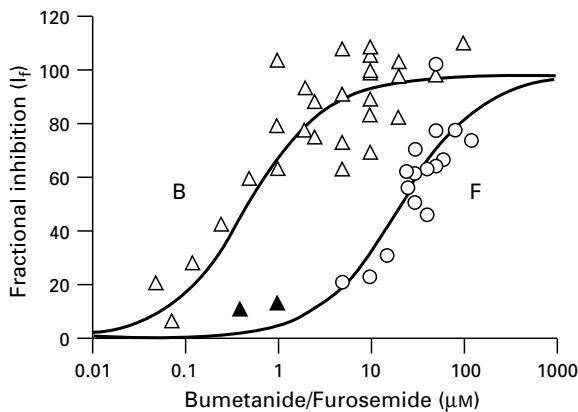
#### Influence of chloride permeability on $V_m$ in control and hypertonic media

To investigate the possible participation of chloride conductance in the response of  $V_m$  to hypertonic shock we used the chloride channel blocker anthracene 9-carboxylic acid (9-AC). In 289 mOsm,  $10 \mu\text{M}$  9-AC gave rise to a small but significant hyperpolarization of  $-2.6 \pm 0.7$  mV ( $n = 4$ ). When medium osmolality was increased to 344 mOsm the response of  $V_m$  to  $10 \mu\text{M}$  9-AC increased significantly to  $-7.6 \pm 0.5$  mV ( $n = 10$ ) ( $P < 0.01$ ) (Figure 4). When more 9-AC was added to this  $10 \mu\text{M}$  concentration (30 or  $70 \mu\text{M}$ ) no additional effect was

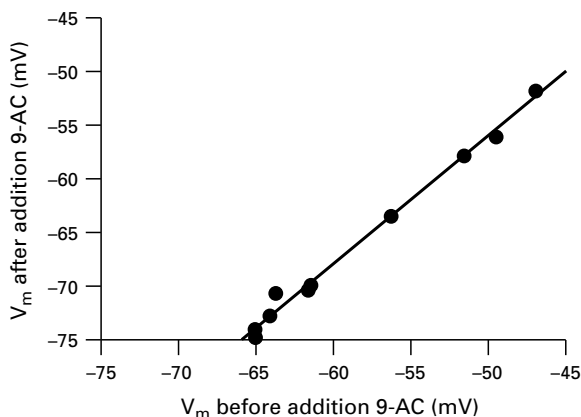
measured. The hyperpolarization found with 9-AC was significantly smaller ( $P < 0.01$ ) than the hyperpolarization of bumetanide. Figure 4 shows that on increasing depolarization by 344 mOsm a significant decrease in 9-AC response was observed and that 9-AC did not restore  $V_m$  to control value with overshoot as was observed for bumetanide.

When 9-AC was added after bumetanide in 344 mOsm no significant change ( $-0.3 \pm 0.3$  mV ( $n = 5$ )) in  $V_m$  was observed. This indicates that chloride in 344 mOsm is in equilibrium when bumetanide is present.

Experiments in media with decreased  $Cl^-$  failed, because  $V_m$  became very unstable in such media.



**Figure 3** The data used to determine the  $IC_{50}$  values for bumetanide and furosemide. The inhibition of the hypertonic (344 mOsm) depolarization by varying concentrations of bumetanide (B) and furosemide (F). The curves were fitted from:  $I_f = 1/(1 + IC_{50}/\text{concentration})$ . Two data points (filled triangles right-below the fitted bumetanide curve at concentrations 0.5 and 1.0  $\mu\text{M}$ ) demonstrating low sensitivity for bumetanide, were both from the same cell. This is the reason why this fit to all data at once produced an  $IC_{50} = 0.49 \mu\text{M}$ . Another procedure which involved first fitting the data of individual cells and then averaging the data of the cells produced an  $IC_{50} = 0.34 \mu\text{M}$ . For furosemide:  $IC_{50} = 21 \mu\text{M}$ . The correlation coefficients,  $r$ , of both fits were 0.83. The  $IC_{50}$  value of bumetanide of 0.5  $\mu\text{M}$  does not comply with the criterion for CCC-2 ( $< 0.2 \mu\text{M}$ ) and is just in the margin of CCC-1 ( $> 0.5 \mu\text{M}$ ) according to the classification of Haas (1994). On close inspection of this figure it might be possible that in our preparation cells with different cotransporters (either CCC-1 or CCC-2) were measured.



**Figure 4** Response of  $V_m$  to anthracene-9-carboxylic acid (9-AC) in 344 mOsm.  $V_m$  after the addition 9-AC was plotted against  $V_m$  before addition of 9-AC. A linear fit gave a good correlation coefficient ( $r = 0.9959$ ) and a slope of just greater than unity ( $V_{m(\text{after})} = 1.2 * V_{m(\text{before})} + 3.6$ ). The slope of  $1.2 \pm 0.04$  is significantly different from 1 ( $P < 0.01$ ), indicating that effect of 9-AC was slightly voltage-dependent.

### Ouabain and bumetanide

Ouabain (100  $\mu\text{M}$ ) depolarized  $V_m$  to  $-50.3 \pm 1.3$  mV ( $n = 6$ ) in 344 mOsm. Application of bumetanide or furosemide, about 10 min after the ouabain addition, did not induce a hyperpolarization. We therefore conclude that a proper functioning of the  $Na^+/K^+-ATPase$  is essential for the bumetanide responses we obtained in this study.

### Discussion

#### Hypertonic stress leads to a sustained depolarization in the skeletal muscle fibre

Skeletal muscle fibres may encounter hypertonicity during exercise (Sjøgaard *et al.*, 1985; Hoffman & Simonsen, 1989). Here we describe the effects of medium osmolality and bumetanide sensitive transport on  $V_m$  in mouse lumbrical muscle fibres. We found this depolarization to rise with increasing hypertonicity in the range of 289 to 344 mOsm, but it exhibited saturation beyond 344 mOsm. The depolarization was maintained as long as the solution was hypertonic. In all cases washing out the PEG restored the  $V_m$  completely even after prolonged exposure.

#### The Na-K-2Cl cotransporter and the depolarization of $V_m$ in hypertonic media

Bumetanide is known to inhibit chloride cotransport such as the K-Cl, Na-Cl and Na-K-2Cl cotransporter (Hoffmann & Simonsen, 1989; Haas, 1994; Lang *et al.*, 1995). In our preparation bumetanide prevented the depolarization by hypertonicity, and repolarized  $V_m$  to control values in hypertonic media. Bumetanide and furosemide appear to act similarly but the fact that the  $IC_{50}$  for bumetanide was much lower than for furosemide suggests that we are not studying the K-Cl cotransporter (Perry & O'Neill, 1993; Haas, 1994). The sensitivity of  $V_m$  to bumetanide need not be a direct measure for the affinity of the cotransporter for bumetanide. These cellular and molecular responses might well differ from one another.

Like bumetanide decreased  $Na^+$  prevented the depolarization by hypertonicity. The concentration of 1.25 mM  $Na^+$  used is substantially lower than the  $K_m$  of  $Na^+$  found for the Na-K-2Cl cotransporter (Greger, 1985). Data on the interaction between hyperosmolality and medium potassium have been presented previously (Siegenbeek van Heukelom *et al.*, 1994). They show that in 0.76 mM  $K^+$  a decreased sensitivity of  $V_m$  for hypertonic stress exists. Due to the presence of the ( $K^+$  sensitive) inwardly rectifying potassium conductance (IKR) a straightforward interpretation is complex. This and the difference in sensitivity of  $V_m$  for bumetanide versus furosemide indicate that we are studying here a  $Cl^-$  related cotransporter where  $Na^+$  and  $K^+$  are involved.

#### Hypertonic medium increases chloride accumulation

Our findings are in line with observations by Aickin *et al.* (1989) that elevated  $Cl^-$  depolarizes  $E_{Cl}$  in rat lumbrical muscle cells. Because  $P_{Cl}$  is 3 to 20 times  $P_K$  (Aickin, 1990), chloride accumulation must have an influence on  $V_m$ . Indeed, by blocking  $P_{Cl}$  we found a hyperpolarization of  $-2.6$  mV in control and  $-7.6$  mV in hypertonic medium, indicating an increased chloride accumulation due to hypertonicity in the lumbrical muscle cells. This apparent dependence on medium osmolality implies that in order to compare 9-AC-induced hyperpolarizations one should take into account the osmolality (Aickin, 1990).

A combined action of the couple  $Na^+/H^+$  and  $Cl^-/HCO_3^-$  or the couple  $Na^+/H^+$  and Na-Cl transport, as was suggested for soleus muscle fibres by Chinnet & Giovannini (1989), ap-

peared not to effect  $V_m$  in our preparation because amiloride had no effect. We conclude that the import through the Na-K-2Cl cotransporter increased with increasing hypertonicity resulting in a depolarization.

#### *$P_{Cl}$ is not zero in the presence of 9-AC*

Like Aickin *et al.* (1989), using furosemide in normo-osmotic medium, we found that addition of 9-AC did not affect  $V_m$  in 344 mOsm medium with bumetanide. We conclude that chloride is in equilibrium across the membrane when the Na-K-2Cl cotransporter is inoperative.

However, the hyperpolarizations induced by 9-AC in 344 mOsm medium were smaller than those induced by bumetanide and did not restore  $V_m$  completely. Comparison of 10  $\mu$ M and 80  $\mu$ M 9-AC indicates that we used a saturating concentration. The most likely explanation is that  $P_{Cl}$  is not fully blocked as has been suggested by Aickin (1990) and that the remaining  $P_{Cl}$  is still large enough to contribute to  $V_m$ , because at the same time chloride is accumulated (Aickin *et al.*, 1989). This increase of intracellular chloride will lead to a further depolarization of  $E_{Cl}$ . So the product of the decreased  $P_{Cl}$  and  $E_{Cl}$  can still have a measurable influence on  $V_m$ .

#### *Potassium permeability is reduced by hypertonicity*

An additional mechanism involved in the depolarization can be seen from the experiments involving pretreatment with bumetanide. On the basis of whole tissue data, Chinet and Giovannini (1989) concluded that rat soleus fibres do not regulate their volume. Cardiac cells showed only partial VRI (Drewnowska & Baumgarten, 1991), like non-mammalian muscle fibres (Blinks, 1965; Mobley & Page, 1971). When the cotransporter is blocked by bumetanide, chloride is passively distributed. Therefore, one would expect a hyperpolarization due to the shrinkage of the cell and hypertonicity ( $K^+$  and  $Na^+$  were increased leading to a hyperpolarization because  $P_K \gg P_{Na}$ ). The simplest way to explain that such hyperpolarization was absent (see Table 1) is to conclude that  $P_K$  drops. If such a decrease in potassium permeability (or conductance) (van Mil *et al.*, 1995a) also occurs in the absence of bumetanide, it will contribute to the observed depolarization

induced by hypertonicity. Such a connection has been suggested also by Wang and Wondergem (1991) for the behaviour of mouse hepatocytes. They suggested that the reduction of  $P_K$  would also reduce the efflux of  $K^+$ -ions as osmolytes.

#### *Hypertonicity affects Na-K-2Cl cotransporter kinetics*

Inhibiting the Na/K-pump with ouabain abolished the dependence of  $V_m$  on medium osmolality, supporting the view that this initially active transport energises the secondary transport studied. This is in line with the decreased dissipation in the presence of bumetanide in soleus muscle fibres observed by Chinet (1993). Drewnowska & Baumgarten (1991) demonstrated that cardiac cells shrink in hypertonic media and that this shrinkage increased in the presence of ouabain. Addition of ouabain induces a depolarization, but this cannot be the reason for the disappearance of the bumetanide induced hyperpolarization. The depolarizations by 465 mOsm to  $-52$  mV, and sometimes by 344 mOsm, can be reversed by bumetanide, although they are the same magnitude as the ouabain-induced depolarizations.

The efflux of water in response to hypertonicity leads to an increase in  $K^+$ ,  $Na^+$  and  $Cl^-$  concentrations in the cell, this reduces the driving force for the import of these ions through the cotransporter. So, the magnitude of the response must be related to a change in transport kinetics and not to the change in ion gradients (Lang *et al.*, 1995). How this is brought about is unclear (Häussinger *et al.*, 1993; 1994; Lang *et al.*, 1995) but the cells may have some 'sensor' for hyperosmotic stress (Galcheva-Gargova *et al.*, 1994), that in our preparation possesses a 'set point' at 269 mOsm.

We conclude that in the lumbrical muscle cells increased hypertonicity leads to an increase in the activity of the Na-K-2Cl cotransporter which results in the observed depolarization of  $V_m$  due to the increased accumulation of  $Cl^-$  together with a decrease in  $P_K$ .

We thank Drs J.A. Groot, M. Joëls, W. van der Laarse and W. Wadman for their critical and constructive comments.

## References

- AICKIN, C.C. (1990). Chloride transport across the sarcolemma of vertebrate smooth and skeletal muscle. In *Chloride Channels and Carriers in Nerve, Muscle, and Glial Cells*. ed. Alvarez-Leefmans, F.J. & Russell, J.M. pp. 209–249. New York, London: Plenum Press.
- AICKIN, C.C., BETZ, W.J. & HARRIS, G.L. (1989). Intracellular chloride and the mechanism for its accumulation in rat lumbrical muscle. *J. Physiol.*, **411**, 437–455.
- BARRY, P.H. & DIAMOND, J.M. (1970). Junction potentials, electrode standard potentials, and other problems in interpreting electrical properties of membranes. *J. Membrane Biol.*, **3**, 93–122.
- BARRY, P.H. & LYNCH, J.W. (1991). Liquid junction potentials and small cell effects in patch-clamp analysis. *J. Membrane Biol.*, **121**, 101–117.
- BLINKS, J.R. (1965). Influence of osmotic strength on cross-section and volume of isolated single muscle fibres. *J. Physiol.*, **177**, 42–57.
- CHINET, A. (1993).  $Ca^{2+}$ -dependent heat production by rat skeletal muscle in hypertonic media depends on Na-Cl co-transport stimulation. *J. Physiol.*, **461**, 689–703.
- CHINET, A. & GIOVANNINI, P. (1989). Evidence by calorimetry for an activation of sodium-hydrogen exchanger of young rat skeletal muscle in hypertonic media. *J. Physiol.*, **415**, 409–422.
- DREWNOWSKA, K. & BAUMGARTEN, C.M. (1991). Regulation of cellular volume in rabbit ventricular myocytes: bumetanide, chlorothiazide and ouabain. *Am. J. Physiol.*, **260**, C122–C131.
- DULHUNTY, A.F. (1978). The dependence of membrane potential on extracellular chloride concentration in mammalian skeletal muscle. *J. Physiol.*, **276**, 67–82.
- GALCHEVA-GARGOVA, Z., DÉRIJARD, B., WU, I.H. & DAVIS, R.J. (1994). An osmosensing signal transduction pathway in mammalian cells. *Science*, **265**, 806–808.
- GORDON, A.M. & GODT, R.E. (1970). Some effects of hypertonic solutions on contraction and excitation-contraction coupling in frog skeletal muscles. *J. Gen. Physiol.*, **55**, 254–275.
- GREGER, R. (1985). Ion transport mechanisms in thick ascending limb of Henle's loop of mammalian nephron. *Physiol. Rev.*, **65**, 760–796.
- HAAS, M. (1994). The Na-K-Cl cotransporters. *Am. J. Physiol.*, **267**, C869–C885.
- HÄUSSINGER, D., GEROK, W. & LANG, F. (1993). Cell volume and hepatic metabolism. In *Advances in Comparative and Environmental Physiology; Vol 14: Interaction of Cell Volume and Cell Function* ed. Lang, F. & Häussinger, D. pp. 33–65. Berlin Heidelberg: Springer-Verlag.
- HÄUSSINGER, D., LANG, F. & GEROK, W. (1994). Regulation of cell function by the cellular hydration state. *Am. J. Physiol.*, **267**, E343–E355.
- HOFFMANN, E.K. & SIMONSEN, L.O. (1989). Membrane mechanisms in volume and pH regulation in vertebrate cells. *Physiol. Rev.*, **69**, 315–382.

- LANG, F., BUSCH, G.L., VÖLKL, H. & HÄUSSINGER, D. (1995). Cell volume: a second message in regulation of cellular function. *News Physiol. Sci.*, **10**, 18–22.
- MOBLEY, B.A. & PAGE, E. (1971). The effect of potassium and chloride ions on the volume and membrane potential of single barnacle muscle cells. *J. Physiol.*, **215**, 49–70.
- PERRY, P.B. & O'NEILL, W.C. (1993). Swelling-activated K fluxes in vascular endothelial cells: volume regulation via K-Cl cotransport and K channels. *Am. J. Physiol.*, **265**, C763–C769.
- SIEGENBEEK VAN HEUKELOM, J. (1991). Role of the anomalous rectifier in determining membrane potentials of mouse fibres at low extracellular K<sup>+</sup>. *J. Physiol.*, **434**, 549–560.
- SIEGENBEEK VAN HEUKELOM, J., VAN MIL, H.G.J., POPTSOVA, M.S. & DOUMAI, R. (1994). What is controlling the cell membrane potential? In *What is Controlling Life? Modern Trends in Biothermokinetics*, Vol. 3. ed. Gnaiger, E. pp. 169–173. Innsbruck, Austria; Innsbruck Univ. Press.
- SJØGAARD, G., ADAMS, R.P. & SALTIN, B. (1985). Water and ion shifts in skeletal muscle of humans with intense dynamic knee extension. *Am. J. Physiol.*, **248**, R190–R196.
- VAN MIL, H.G.J., GEUKES FOPPEN, R.J. & SIEGENBEEK VAN HEUKELOM, J. (1995a). Bumetanide suppresses skeletal muscle membrane potential changes induced by hypertonic media. *Pflügers Arch.*, **430**, R55.
- VAN MIL, H.G.J., KERKHOF, C.J.M. & SIEGENBEEK VAN HEUKELOM, J. (1995b). Modulation of the isoprenaline-induced membrane hyperpolarization of mouse skeletal muscle cells. *Br. J. Pharmacol.*, **116**, 2881–2888.
- WANG, K. & WONDERGEM, R. (1991). Effects of hyperosmotic medium on hepatocyte volume, transmembrane potential and intracellular K<sup>+</sup> activity. *Biochim. Biophys. Acta*, **1069**, 187–196.
- WEINER, I.M. & MUDGE, G.H. (1990). Diuretics and other agents employed in the mobilization of edema fluid. In *Goodman and Gillman's Pharmacological Basis of Therapeutics*. ed. Goodman Gillman, A., Rall, T.W., Nies, A.S. & Taylor, P. pp. 713–731. Oxford: Pergamon Press.

(Received May 31, 1996  
Revised September 4, 1996  
Accepted October 3, 1996)