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Hyperpolarization of the cell membrane of mouse hepatocytes by lactate, pyruvate, and fructose is due to Ca^{2+}-dependent activation of K^+ channels and of the Na^+/K^+-ATPase

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Abstract

Using superfused mouse liver slices combined with a conventional microelectrode technique, we investigated: (1) the ionic mechanisms involved in the hyperpolarization of the hepatocyte membrane induced by lactate and other gluconeogenic substrates; (2) whether these mechanisms are similar to those underlying the hyperpolarization induced by cell swelling in hypo-osmotic medium; and (3) whether the hyperpolarizing effect of lactate on the hepatocyte membrane is related to gluconeogenesis. Lactate (5 mmol/l) hyperpolarized the hepatocyte membrane after an exposure of 10–20 min, and the hyperpolarization was still present after 70 min. The hyperpolarization induced by lactate, pyruvate (5 mmol/l) and fructose (10 mmol/l), and by exposure to hypo-osmotic medium (250 mosmol/l) was antagonized by ouabain, tetraethylammonium (TEA), and cetiedil (lactate; hypo-osmotic medium). Hyperpolarization induced by lactate was eliminated or attenuated by agents impairing activation of Ca^{2+}-dependent K^+ channels, by amiloride, and by a blockade of non-selective cation channels with flufenamic acid and gadolinium. Thapsigargin, increasing cytosolic Ca^{2+}, mimicked lactate's hyperpolarizing effect. Lactate's effect was dependent on extracellular Ca^{2+}. Finally, lactate's hyperpolarizing effect was reduced by inhibiting gluconeogenesis. These findings suggest that metabolism of lactate hyperpolarizes hepatocytes by mechanisms analogous to those underlying the hyperpolarization induced by cell swelling in hypo-osmotic medium. Gluconeogenesis from lactate may cause cell swelling, subsequent activation of Ca^{2+}-dependent K^+ channels and of the Na^+/K^+-ATPase, and thus hyperpolarize the hepatocyte membrane.

Keywords: Hepatocyte; Hyperpolarization; Lactate; Hypo-osmotic medium; Ca^{2+}; Malignancy-associated hypercalcemia; Hyperlactemia

1. Introduction

It has been reported that in the perfused rat liver, stimulation of gluconeogenesis from lactate by glucagon is correlated with a hyperpolarization of the liver cell membrane [1]. Insulin, a hormone known to antagonize the effect of glucagon on gluconeogenesis, blocked the hyperpolarizing effect of glucagon [1]. Glucose by itself had no long-term effect on the liver cell membrane potential [2,3]. It has also been observed that gluconeogenic substrates (e.g. lactate, pyruvate, fructose) are able to hyperpolarize the hep-

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atocyte membrane even in the absence of hormones [2,3]. The aim of the present study was to investigate whether various agents inhibiting gluconeogenesis antagonize lactate's hyperpolarizing effect on the liver cell membrane. Positive results would support the hypothesis that lactate induced hyperpolarization is due to lactate metabolism in particular by gluconeogenesis. A further intention was to investigate the ionic mechanism of the hyperpolarization induced by the gluconeogenic substrates lactate, pyruvate, and fructose. Since the electrogenic Na^+/K^+-ATPase and the membrane K^+ conductance are both important determinants of the hepatic membrane potential [4], the effects of ouabain, an inhibitor of the Na^+/K^+-ATPase, and of various K^+ channel blockers on gluconeogenic substrate induced hyperpolarization were tested. Moreover, we compared the effect of lactate on the hepatic membrane potential with that of a hypo-osmotic medium to find out whether cell swelling might be involved in the hyperpolarization induced by lactate and other gluconeogenic substrates.

The investigations are potentially related to the hepatic control of feeding because lactate, pyruvate and fructose seem to inhibit feeding by acting on the liver [5–8]. Furthermore, the membrane potential of liver cells has been postulated to represent a major signal in the control of feeding [9].

2. Materials and methods

2.1. Animals and maintenance

Adult female mice (Zur:ICR) with a body weight of 30–50 g were used for the experiments. Mice were fed ad libitum with a diet containing 18% fat, 46% carbohydrate and 13% protein [3]. Mice were adapted to this diet for at least 2 weeks and had ad libitum access to food and water until the experiment.

2.2. Liver slice preparation, maintenance and temperature control

Mice were killed by cervical dislocation and the median lobe of the liver was removed quickly and placed on gauze moistened with modified Krebs–Henseleit bicarbonate buffer (see below). The liver lobe was fixed by applying slight pressure with a glass microscope slide while preparing liver slices (5×5 mm; thickness about 1 mm) with a scalpel blade. After incubation for 40–100 min (mean 70 min) in oxygenated (95% O_2, 5% CO_2) control or experimental medium (37°C), the liver slices were placed into an acrylic chamber with the encapsulated uncut surface of the liver slice upward. All microelectrode impalements were of superficial cells on the uncut surface. Viability of this liver slice preparation has been evaluated previously by Wondergem and Castillo [10] who measured similar membrane potentials using superfused mouse liver slices or mouse hepatocytes in primary monolayer culture prepared from whole liver.

The liver slices were superfused at a rate of about 12 ml/min with an oxygenated Krebs–Henseleit bicarbonate buffer (medium; containing (mmol/l): NaCl 103; KCl 4.7; $CaCl_2$ 2.56; $MgCl_2$ 1.3; $NaHCO_3$ 25; NaH_2PO_4 1.15). In the experimental solution, part of NaCl was replaced equiosmotically with Na-lactate (5 mmol/l), Na-pyruvate (5 mmol/l), fructose (10 mmol/l), glucose (10 mmol/l), α-cyano-4-hydroxycinnamate (CHC; 5 mmol/l), or perfluorosuccinate (PFS; 10 mmol/l). Part of the experiments were performed using superfusion solutions with reduced Ca^{2+} (1.28 mmol/l) and Mg^{2+} (0.65 mmol/l) concentrations or under Ca^{2+}-free conditions. In these cases, $CaCl_2$ or $MgCl_2$ were replaced equiosmotically with NaCl. The medium for incubation prior to superfusion generally had the same composition as the superfusion solutions except in the experiment examining the time course of lactate's effect on hepatocyte membrane potential (see below).

In some experiments, the influence of cell swelling induced by hypo-osmotic superfusion medium on the membrane potential of hepatocytes was examined. In these experiments, NaCl was partly (25 mmol/l) replaced by D(+)-cellobiose (50 mmol/l) in the control solution (iso-osmotic). Cell swelling was then induced by superfusion with the experimental solution in which D(+)-cellobiose was omitted (hypo-osmotic; 250 mosmol/l).

The buffer temperature was maintained at 37°C and monitored continuously with a thermistor (Ebro CTA 1220; Ingoldstadt, Germany).

2.3. Fabrication of open-tip microelectrodes

Open-tip microelectrodes were drawn in a horizontal puller (Sachs-Fleming Micropipette Puller PC-84; Sutter Instrument, San Raphael, CA, USA) from microfilament glass capillaries (1.5 mm o.d., 0.86 mm i.d.; A-M Systems, Everett, WA, USA). Pipettes were filled with KCl (0.5 mol/l).

2.4. Measurement of membrane potential (V_m)

The microelectrode was connected by an Ag–AgCl half cell to a high input impedance preamplifier (10^{13} Ω; Biologic VF 180; Echirolles, France). The reference electrode was connected by an Ag–AgCl half cell to the tissue chamber by an agar (4% in Krebs–Henseleit buffer) bridge. Voltage was measured with a digital voltmeter and an oscilloscope (Kikusui COS 5020; Kawasaki City, Japan) and recorded on a two-channel recorder (Rikadenki B-281-L; Kogyo, Japan). Criteria for valid micropipette impalements of liver cells were: (1) a rapid deflection of the voltage trace on advancing the microelectrode into the liver slice; (2) a stable voltage trace within 2 mV for at least 30 s; and (3) return of the voltage trace to within 2 mV of the baseline when the microelectrode was withdrawn. Resistance of open-tip microelectrodes (20–50 MΩ) was measured once before every impalement by passing AC pulses ($I = 1$ nA; frequency 1000 Hz). For every liver slice, the membrane potential of 5–6 liver cells was measured, and each recording of membrane potential lasted at least 30 s.

2.5. Standard experimental protocol

Three to eight mice were used for one experiment. The liver lobes of each mouse were fragmented into 8 slices with slices 1–4 placed in control and slices 5–8 placed in experimental medium for incubation (see above). Then, the membrane potential (V_m) was measured in the order from slices 1 to 8. The order was alternated from slices 8 to 1 for the next mouse. This allowed a mean incubation time of approximately 70 min in both control and experimental slices. Preliminary experiments had shown that membrane potential was stable between 40 and 100 min after the start of incubation (results not shown). The influence of a time delay between the start and the end of an individual experiment is, therefore, considered negligible.

2.6. Measurement of time course

The influence of K^+ channel blockers and ouabain on the hyperpolarization induced by lactate or hypotonic medium was also examined during short-term superfusion. In these experiments, the membrane potential of hepatocytes was measured 5 and 10 min after preparation of liver slices (superfusion medium 1, Krebs–Henseleit buffer without channel blockers or ouabain; superfusion medium 2, Krebs–Henseleit buffer with channel blockers or ouabain). Then, the superfusion solutions were switched to the respective solutions (without or with blockers) containing lactate, or the hypo-osmotic solutions, respectively.

2.7. Chemicals used

Sources of chemicals: Na-lactate, Na-pyruvate, fructose, D-glucose, tetraethylammonium chloride (TEA), quinine hydrochloride, gadolinium chloride, were all from Fluka Chemie (Buchs, Switzerland); D(+)-cellobiose, ouabain, amiloride hydrochloride, α-cyano-4-hydroxycinnamate (CHC), apamin, iberiotoxin, verapamil, thapsigargin, flufenamic acid, and glibenclamide were all from Sigma (Buchs, Switzerland); insulin from Novo Nordisk (Küsnacht, Switzerland); perfluorosuccinate (PFS) from Pfaltz and Bauer (Waterbury, CT, USA); cetiedil from Inothera (Arcueil, France); and $BaCl_2$ from Siegfried (Zofingen, Switzerland). Glibenclamide was dissolved in dimethylsulfoxide (DMSO; Sigma) prior to addition to the medium (0.5 ml DMSO for 1 l of medium).

2.8. Statistical evaluation

All values are presented as mean ± S.E. For the experiments using long-term incubation of liver slices, membrane potentials from all liver cells measured under experimental or control conditions (5–6 cells within each liver slice, 8 liver slices per mouse) were combined to form a pair of mean experimental or control values. These mean values were considered representative for the respective mouse. Differences

between treatments were statistically evaluated using the paired Student's *t*-test (n = number of mice).

Differences in the hyperpolarizing effect observed under different conditions, i.e. differences in the membrane potential measured with the experimental solution compared with the respective control medium, were evaluated using the unpaired Student's *t*-test or using ANOVA with the Student–Newman–Keuls post hoc test if more than two groups were compared. When investigating the time course of the hyperpolarizing effect of lactate or hypotonic medium, repeated measures ANOVA was used. Differences between control and experimental conditions at each individual time point were evaluated using an unpaired Student's *t*-test.

In all cases, a P-value < 0.05 was considered significant.

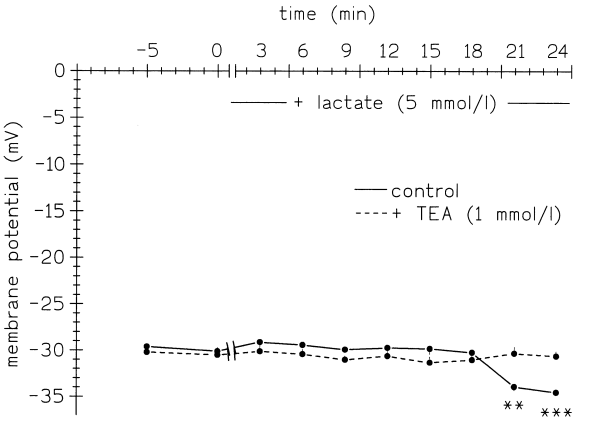


Fig. 1. Influence of the K^+ channel blocker tetraethylammonium (TEA; 1 mmol/l) on the hyperpolarization induced by superfusion of mouse liver slices with lactate (5 mmol/l). Superfusion with Krebs–Henseleit buffer from $t = -10$ to $t = 0$ min. Then, lactate was added. $n = 8$; $^{**}P < 0.01$; $^{***}P < 0.001$ (unpaired Student's *t*-test).

3. Results

Lactate (5 mmol/l) significantly hyperpolarized the membrane of mouse hepatocytes by approximately 4–5 mV during a mean exposure of 70 min (Tables 1–3). The hyperpolarizing effect of lactate was abolished by glucose (10 mmol/l), insulin (10^{-7} mol/l), α-cyano-4-hydroxycinnamate (CHC; 5 mmol/l), which inhibits gluconeogenesis by blocking channeling of pyruvate in the gluconeogenic pathway [11], and perfluorosuccinate (PFS; 10 mmol/l), an inhibitor of phospho-enol-pyruvate carboxykinase, a key enzyme in gluconeogenesis [12] (Table 1). CHC alone produced a marked hyperpolarization ($P < 0.001$; CHC vs. absence of CHC; Table 1).

Ouabain (1 mmol/l) neutralized the hyperpolarizing effects of lactate, pyruvate and fructose on the liver cell membrane (Table 2). The K^+ channel blocker TEA (1 mmol/l) attenuated these effects (Table 2). The hyperpolarizing effect of fructose remained unaffected by CHC, which itself again produced a marked hyperpolarization (Table 2). The influence of TEA and ouabain on the hyperpolarizing effect of lactate under short-term conditions was similar to that under long-term exposure (70 min). While TEA and ouabain had no effect on basal

Table 1
Modulation of the hyperpolarizing effect (d mV) of lactate (5 mmol/l) on the cell membrane of mouse hepatocytes by glucose, insulin, α-cyano-4-hydroxy-cinnamate (CHC) and perfluorosuccinate (PFS)

	Membrane potential (mV)		
	Control	+Lactate (5 mmol/l)	d mV[a]
Krebs–Henseleit buffer ($n = 5$)[b]	−29.6 ± 0.8	−33.5 ± 0.3**	3.8 ± 0.6
+glucose (10 mmol/l) ($n = 5$)	−32.6 ± 1.2	−32.5 ± 0.5	−0.1 ± 1.0[#]
+insulin (10^{-7} mol/l) ($n = 5$)	−31.4 ± 0.3	−32.1 ± 0.5	0.7 ± 0.6[#]
+CHC (5 mmol/l) ($n = 4$)	−41.5 ± 1.2	−40.9 ± 1.7	−0.6 ± 1.6
+PFS (10 mmol/l) ($n = 4$)	−26.0 ± 0.4	−25.8 ± 0.2	−0.2 ± 0.3[#]

[a]Difference between experimental and control conditions.
[b]n = number of animals.
**Significantly different from control value ($P < 0.01$).
[#]Significantly different from the hyperpolarizing effect of lactate alone ($P < 0.05$).

Table 2
Modulation of the hyperpolarizing effect (d mV) of lactate (5 mmol/l), pyruvate (5 mmol/l), and fructose (10 mmol/l) on the cell membrane of mouse hepatocytes by ouabain (1 mmol/l), tetraethylammonium (TEA; 1 mmol/l), and α-cyano-4-hydroxycinnamate (CHC; 5 mmol/l)

	Membrane potential (mV)		
	Control	+Lactate (5 mmol/l)	d mV[b]
Krebs–Henseleit buffer[a] ($n=4$)[c]	−31.5 ± 0.7	−35.2 ± 0.8**	3.7 ± 0.6
+ouabain (1 mmol/l) ($n=6$)	−29.2 ± 0.7	−30.0 ± 0.6	0.8 ± 0.6[#]
+TEA (1 mmol/l)[a] ($n=4$)	−31.2 ± 0.5	−32.6 ± 0.6	1.4 ± 0.7[#]
	Control	+Pyruvate (5 mmol/l)	d mV
Krebs–Henseleit buffer ($n=4$)	−28.9 ± 0.7	−34.1 ± 0.6**	5.2 ± 0.7
+ouabain (1 mmol/l) ($n=4$)	−27.7 ± 0.6	−28.2 ± 0.2	0.5 ± 0.5[##]
+TEA (1 mmol/l) ($n=3$)	−29.4 ± 0.5	−32.3 ± 0.9	2.9 ± 0.7
	Control	+Fructose (10 mmol/l)	d mV
Krebs–Henseleit buffer ($n=5$)	−30.3 ± 0.5	−33.7 ± 0.4**	3.5 ± 0.7
+ouabain (1 mmol/l) ($n=4$)	−30.2 ± 0.6	−30.1 ± 0.4	−0.1 ± 0.8[#]
+TEA (1 mmol/l) ($n=5$)	−30.8 ± 0.5	−31.6 ± 0.4	0.8 ± 0.6[#]
+CHC (5 mmol/l) ($n=6$)	−42.8 ± 1.6	−46.2 ± 1.5*	3.4 ± 0.9

[a]Values have already been published in Rossi and Scharrer [3].
[b]Difference between experimental and control conditions.
[c]n = number of animals.
*,**Significantly different from control value ($P<0.05$ or $P<0.01$, respectively).
[#,##]Significantly different from the hyperpolarizing effect of respective substrate alone ($P<0.05$ or $P<0.01$, respectively).

membrane potential, they completely abolished lactate's hyperpolarizing effect during short-term exposure (Figs. 1 and 2).

Amiloride (1 mmol/l) significantly ($P<0.05$) reduced the hyperpolarizing effect of lactate (Table 3). Quinine, Ba^{2+} and verapamil either abolished (quinine) or markedly reduced ($P<0.05$) the hyperpolarizing effect of lactate (Table 3). Apamin (40 nmol/l), a blocker of a subclass of Ca^{2+}-activated K^+ channels with low conductance [13,14], also significantly lowered lactate's effect, whereas the inhibition by iberiotoxin was not significant. A blocker of

Table 3
Modulation of the hyperpolarizing effect (d mV) of lactate (5 mmol/l) on the cell membrane of mouse hepatocytes by amiloride, quinine, iberiotoxin, verapamil, $BaCl_2$, apamin, or glibenclamide

	Membrane potential (mV)		
	Control	+Lactate (5 mmol/l)	d mV[a]
Krebs–Henseleit buffer ($n=4$)[b]	−30.4 ± 0.7	−35.5 ± 1.2*	5.1 ± 1.0
+amiloride (1 mmol/l) ($n=4$)	−28.4 ± 0.5	−30.3 ± 0.6*	2.0 ± 0.5[#]
+quinine (1 mmol/l) ($n=5$)	−21.7 ± 2.0	−19.5 ± 1.8	−2.2 ± 1.7[#]
+iberiotoxin (5 nmol/l) ($n=4$)	−30.1 ± 1.1	−32.9 ± 1.1	2.8 ± 1.3
+verapamil (100 μmol/l) ($n=8$)	−26.9 ± 0.6	−28.8 ± 0.8*	1.9 ± 0.6[#]
+$BaCl_2$ (5 mmol/l) ($n=4$)	−24.3 ± 2.0	−25.6 ± 1.7	1.3 ± 1.2[#]
+apamin (40 nmol/l) ($n=4$)	−30.7 ± 1.0	−32.7 ± 0.9*	2.0 ± 0.4[#]
+glibenclamide (30 μmol/l) ($n=4$)	−27.7 ± 0.2	−32.1 ± 0.3**	4.4 ± 0.4

[a]Difference between experimental and control conditions.
[b]n = number of animals.
*,**Significantly different from control value ($P<0.05$ or $P<0.01$, respectively).
[#]Significantly different from the hyperpolarizing effect of lactate alone ($P<0.05$).

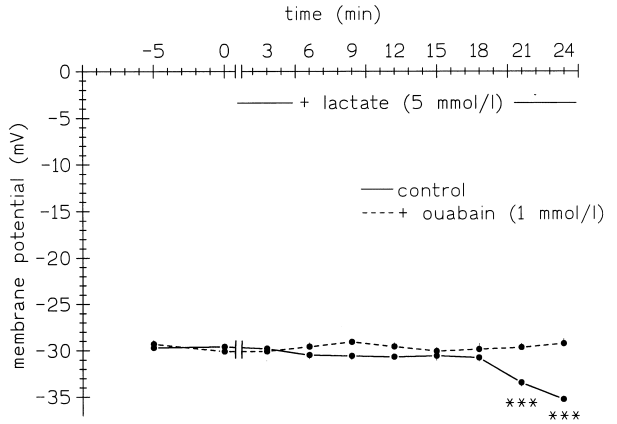


Fig. 2. Influence of the blocker of the Na^+/K^+ATPase ouabain (1 mmol/l) on the hyperpolarization induced by superfusion of mouse liver slices with lactate (5 mmol/l). Superfusion with Krebs–Henseleit buffer from $t=-10$ to $t=0$ min. Then, lactate was added. $n=8$; $^{***}P<0.001$ (unpaired Student's t-test).

ATP-sensitive K^+ channels, glibenclamide (30 μmol/l [14]), had no effect on the hyperpolarization induced by lactate after a mean exposure of 70 min (Table 3). Flufenamic acid, a blocker of non-selective cation channels [15], abolished lactate's effect (Fig. 3). Similar results were obtained with gadolinium (10 μmol/l), another blocker of non-selective ion channels [16,17].

Because lactate's hyperpolarizing effect seems to be linked to gluconeogenesis (Table 1), and since glucose production from lactate and other gluconeogenic substrates might lead to osmotic cell swelling, we tested whether hyperpolarization induced by cell swelling due to exposure to hypo-osmotic medium (about 250 mosmol/l) is similarly affected by ion channel blockers and ouabain. Indeed, the hyperpolarization induced by hypo-osmotic medium (Figs. 4 and 5) was abolished by cetiedil, which also blocked lactate's hyperpolarizing effect (Fig. 4), by TEA, ouabain (Fig. 5), and the blockers of non-selective ion channels, flufenamic acid (100 μmol/l) and gadolinium (10 μmol/l) [15–17].

Since Ca^{2+} activated K^+ channels seem to be involved in the hyperpolarizing effect of lactate (Table

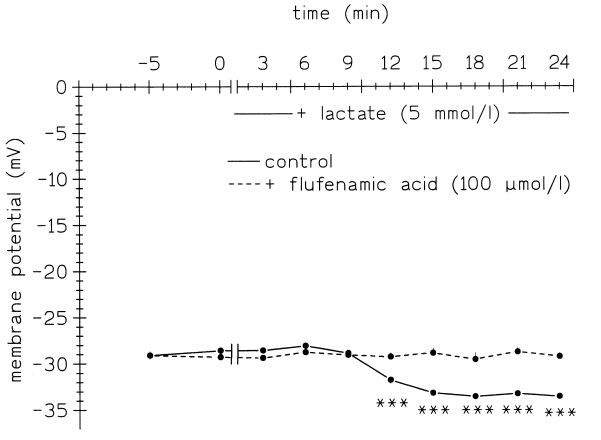


Fig. 3. Influence of the blocker of non-selective cation channels flufenamic acid (100 μmol/l) on the hyperpolarization induced by superfusion of mouse liver slices with lactate (5 mmol/l). Superfusion with Krebs–Henseleit buffer from $t=-10$ to $t=0$ min. Then, lactate was added. $n=8$; $^{***}P<0.001$ (unpaired Student's t-test).

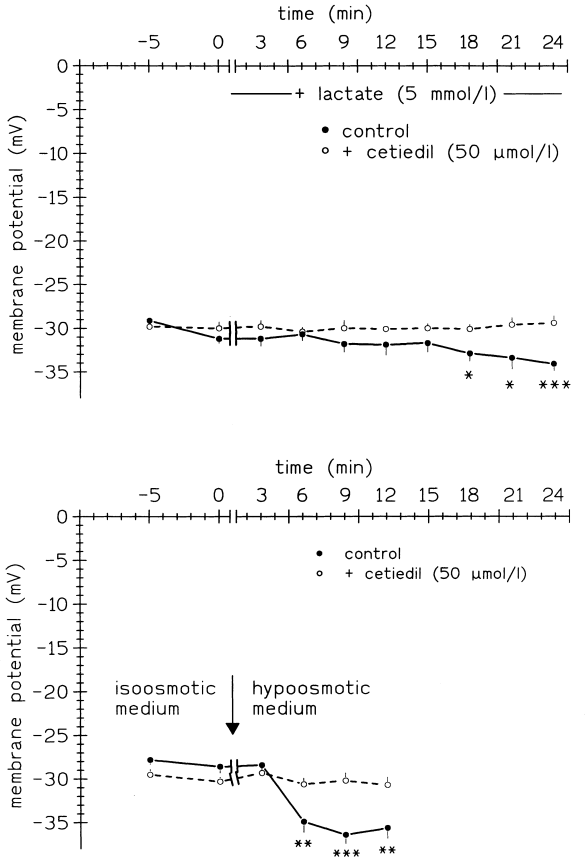
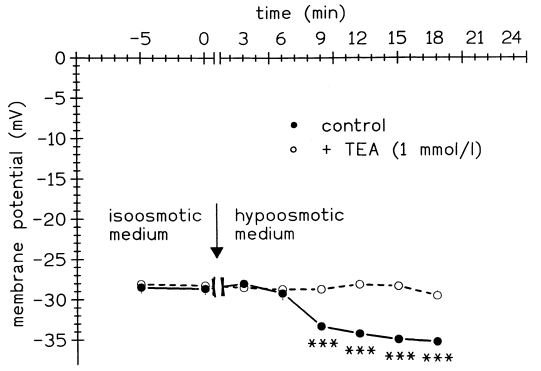


Fig. 4. Influence of the K^+ channel blocker cetiedil (50 μmol/l) on the hyperpolarization induced by superfusion of mouse liver slices with lactate (5 mmol/l) or exposure to hypo-osmotic medium (250 mosmol/l). Superfusion with iso-osmotic Krebs–Henseleit buffer (300 mosmol/l) from $t=-10$ to $t=0$ min. Then, lactate was added, or superfusion continued with hypo-osmotic medium. $n=8$; $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$ (unpaired Student's t-test).



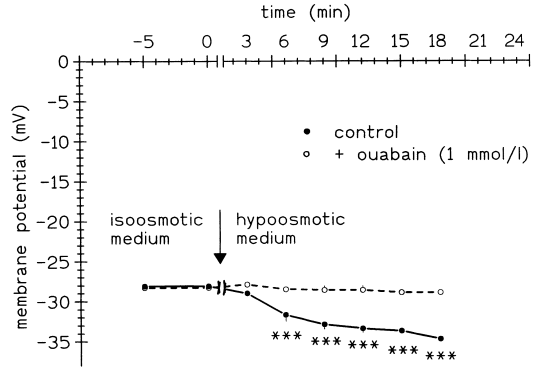


Fig. 5. Influence of TEA (1 mmol/l) and ouabain (1 mmol/l) on the hyperpolarization of the liver cell membrane induced by exposure to hypo-osmotic medium (250 mosmol/l). Superfusion with iso-osmotic Krebs–Henseleit buffer (300 mosmol/l) from $t=-10$ to $t=0$ min. Then, superfusion continued with hypo-osmotic medium. $n=8$; $^{***}P<0.001$ (unpaired Student's t-test).

3), we also tested whether a modulation of the cytosolic or extracellular Ca^{2+} concentration influences

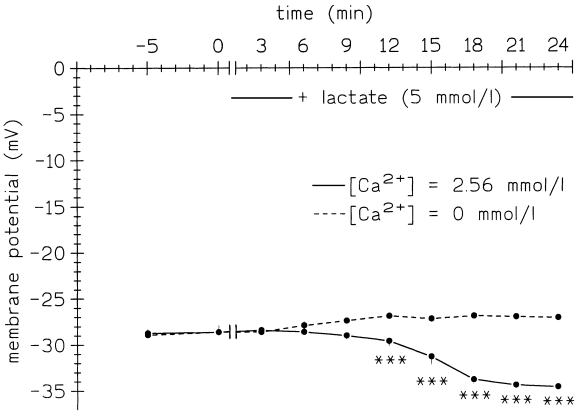


Fig. 6. Influence of the presence of Ca^{2+} in the superfusion medium on the hyperpolarization induced by lactate (5 mmol/l). Superfusion with Krebs–Henseleit buffer from $t=-10$ to $t=0$ min. Then, lactate was added. $n=8$; $^{***}P<0.001$ (unpaired Student's t-test).

this effect. Thapsigargin (1 μmol/l), which inhibits the endoplasmic reticulum Ca^{2+}-ATPase and thus increases the cytosolic Ca^{2+} concentration [18], significantly hyperpolarized the liver cell membrane (Table 4). Thapsigargin's hyperpolarizing effect was abolished by TEA and ouabain (Table 4). Under nominally Ca^{2+}-free conditions, lactate (5 or 20 mmol/l) did not hyperpolarize the liver cell membrane (Fig. 6). Similarly, lactate (5 mmol/l) induced only a slight (basal, -28.9 ± 0.2 vs. -30.5 ± 0.4 mV after 24 min superfusion with lactate) non-significant hyperpolarization of the hepatocyte membrane at a reduced Ca^{2+} concentration (1.28 mmol/l compared with 2.56 mmol/l) in the superfusion medium. Increasing the lactate concentration to 20 mmol/l resulted in a significant hyperpolarization at a Ca^{2+} concentration of 1.28 mmol/l (Mg^{2+}, 0.65 mmol/l)

Table 4
Modulation of the hyperpolarizing effect (d mV) of thapsigargin (1 μmol/l) on the cell membrane of mouse hepatocytes by ouabain (1 mmol/l), and tetraethylammonium (TEA; 1 mmol/l)

	Membrane potential (mV)		
	Control	+Thapsigargin (1 μmol/l)	d mV[a]
Krebs–Henseleit buffer ($n=8$)[b]	-28.4 ± 0.4	-31.7 ± 0.4**	3.3 ± 0.6
+ouabain (1 mmol/l) ($n=4$)	-29.2 ± 0.1	-29.3 ± 0.1	0.1 ± 0.1[#]
+TEA (1 mmol/l) ($n=4$)	-29.9 ± 0.7	-30.9 ± 0.5	1.0 ± 0.9[#]

[a]Difference between experimental and control conditions.
[b]n = number of animals.
**Significantly different from control value ($P<0.01$).
[#]Significantly different from the hyperpolarizing effect of thapsigargin alone ($P<0.05$).

(basal value compared with 21 min after superfusion with lactate: Ca^{2+} 2.56 mmol/l, -29.8 ± 0.4 vs. -34.4 ± 0.3 ($P < 0.001$); Ca^{2+} 1.28 mmol/l, -29.1 ± 0.3 vs. -33.7 ± 0.5 ($P < 0.001$)).

4. Discussion

The present study shows, that the hyperpolarization induced by lactate or hypo-osmotic medium was abolished by the K^+ channel blockers TEA and cetiedil and by the inhibitor of the Na^+/K^+-ATPase, ouabain. A blockade of non-selective cation channels with flufenamic acid and gadolinium also eliminated the lactate-induced hyperpolarization, which was dependent on extracellular Ca.

The hyperpolarization of the liver cell membrane by lactate, pyruvate, and fructose basically confirms previous findings [2,3,19]. The mechanisms underlying this hyperpolarizing effect appear to be analogous to those underlying the hyperpolarizing effect of hypo-osmotic medium because TEA, cetiedil, ouabain, flufenamic acid and gadolinium abolished the hyperpolarization induced both by lactate and by hypo-osmotic medium. Since the latter effect is due to cell swelling [20], it is likely that lactate induced hyperpolarization also occurs subsequently to cell swelling. This idea agrees with the hypothesis that lactate induced hyperpolarization of the liver cell membrane might be produced by gluconeogenesis [1,19] because after its uptake into liver cells [21], lactate is quickly metabolized mainly to glucose [22] which could increase the intracellular osmolarity leading to water influx and cell swelling. Metabolism of lactate within the liver cells would in addition enhance its intracellular uptake by maintaining a concentration gradient between the extracellular and intracellular space.

The attenuation of the hyperpolarizing effect of lactate by inhibiting gluconeogenesis with various agents such as glucose [23], insulin [24,25], α-cyano-4-hydroxycinnamate (CHC [11]), or perfluorosuccinate (PFS [12]) also corroborated the involvement of gluconeogenesis in this effect. The lack of effect of insulin on the membrane potential in the absence of a gluconeogenic substrate also suggests that insulin's effect on the membrane potential in the presence of lactate is related to inhibition of gluconeogenesis. CHC inhibits pyruvate transport across the cell membrane and the mitochondrial membrane and thus blocks channeling of pyruvate in the gluconeogenic pathway [11], and PFS is an inhibitor of phospho-enol-pyruvate carboxykinase, a key enzyme in gluconeogenesis [12]. CHC had no effect, however, on the hyperpolarization induced by fructose. This agrees with our assumption of lactate's hyperpolarizing effect being linked to gluconeogenesis since fructose is channeled into the gluconeogenic pathway beyond the step inhibited by CHC. Since fructose hyperpolarized the hepatocyte membrane despite higher baseline levels due to CHC, it is unlikely that the hyperpolarization induced by CHC itself can explain the lack of effect of lactate on liver cell membrane potential in the presence of CHC.

Since CHC inhibits pyruvate transport across the mitochondrial membrane [11], it not only reduces its channeling into the gluconeogenic pathway, but also oxidation by pyruvate dehydrogenase and oxidative phosphorylation, and thus reduces production of ATP. The subsequent ATP depletion may increase the cytosolic Ca^{2+} concentration due to a lower activity of the plasma membrane and endoplasmic reticulum Ca^{2+}-ATPases [26]. The final consequence could be opening of Ca^{2+}-activated K^+ channels and thus hyperpolarization of the hepatocyte membrane. Since 2,5-anhydro-D-mannitol, which depletes hepatocytes of ATP, hyperpolarized the hepatocyte membrane by opening apamin-sensitive Ca^{2+}-activated K^+ channels [27], it may be hypothesized that these channels are also involved in CHC's hyperpolarizing effect. CHC also competitively inhibits lactate uptake across the cell membrane [11], so that CHC's antagonistic effect on lactate induced hyperpolarization might partly be due to inhibition of lactate transport through the liver cell membrane.

The hyperpolarization of the liver cell membrane induced by lactate, pyruvate, fructose and thapsigargin, and by superfusion with hypo-osmotic medium was prevented by ouabain and the K^+ channel blockers TEA and cetiedil (lactate, hypo-osmotic medium). It therefore appears likely, that activation of the Na^+/K^+-ATPase and of K^+ channels is functionally coupled. The underlying mechanism of this coupling [28] remains to be investigated. It is plausible that opening of K^+ channels and K^+ efflux subsequent to cell swelling activates the Na^+/K^+-ATPase.

This could lead to a reuptake of K^+, an extrusion of Na^+ from the cells, and an enhanced transfer of Cl^- into the extracellular space resulting from membrane hyperpolarization. Reuptake of K^+ would also prevent a decrease in intracellular K^+ due to cell swelling [29]. This coupling of K^+ channels, the Na^+/K^+-ATPase and secondary Cl^- efflux, leading overall to Na^+ and Cl^- efflux from the cells, could participate in the regulatory volume decrease (RVD [20,30]) partly counteracting cell swelling. In agreement with our results, other studies had shown that blockade of K^+ channels prevents the hyperpolarization of the liver cell membrane in hypo-osmotic medium [20,31]. Previous findings [29], however, argued against an involvement of the Na^+/K^+-ATPase in the RVD of mouse liver cells since ouabain did not prevent the hyperpolarization induced by hypo-osmotic medium in the study by Wang and Wondergem [29]. It is unknown at present if the conflicting outcome can be explained by different experimental conditions since in their study [29], the superfusion medium contained metabolizable substrates, such as glucose, pyruvate, glutamate and fumarate, which could have caused stimulation of the Na^+/K^+-ATPase not being increased further by hypo-osmotic medium.

Exposure of liver slices to ouabain had no or only a minor depolarizing effect on hepatocytes under control conditions although ouabain prevented the hyperpolarization induced by lactate, pyruvate, fructose, thapsigargin and hypo-osmotic medium. Hepatocyte membrane potential in the basal state therefore does not seem to depend substantially on the action of the ouabain-sensitive Na^+/K^+-ATPase under our experimental conditions. Under stimulated conditions, however, such as in the presence of lactate or during hypo-osmotic stress, which appear to increase K^+ conductivity, it seems to contribute to the observed hyperpolarization.

Interestingly, similar to the hyperpolarization of the mouse liver cell membrane induced by cell swelling in hypo-osmotic (210 vs. 280 mosmol/l) medium [32], lactate's hyperpolarizing effect was also dependent on the extracellular Ca^{2+} concentration. In addition, agents impairing the activation of Ca^{2+}-dependent K^+ channels, i.e. quinine, verapamil, Ba^{2+}, apamin [14,31,33] and cetiedil, which in addition to volume-sensitive K^+ channels [31] also blocks Ca^{2+}-dependent K^+ channels at higher concentrations [34], attenuated or abolished lactate's effect. It is, therefore, likely that the activation of K^+ channels subsequent to cell swelling mainly involves Ca^{2+}-dependent K^+ channels [30]. It has to be kept in mind, however, that the hyperpolarizing effect induced by hypo-osmotic medium appears to be less sensitive to low Ca^{2+} concentrations in the superfusion medium than lactate's effect since the former effect was only blocked when in addition to using Ca^{2+}-free superfusion buffer, EGTA (ethylene glycol-bis (β-aminoethylether)-*N*,*N*′-tetraacetic acid) was added to the solution to sequester free extracellular Ca^{2+} in the glycocalyx [32]. Further support for the hypothesis of a major role of Ca^{2+}-dependent K^+ channels is brought about by the hyperpolarizing effect of thapsigargin, which increases the intracellular Ca^{2+} concentration by a selective inhibition of the Ca^{2+}-ATPase of the endoplasmic reticulum [18], that similar to lactate's hyperpolarizing effect, was also prevented by ouabain and TEA.

The findings presented in this study are consistent with the assumption that gluconeogenesis, leading to cell swelling, hyperpolarizes the liver cell membrane by an increase in cytosolic Ca^{2+} being associated with the activation of the Na^+/K^+-ATPase and K^+ channels. Previous studies have documented the presence of non-selective cation channels in hepatocytes which are permeable to Na^+, K^+, and Ca^{2+}, and their activation by cell swelling [30,35]. Since flufenamic acid and gadolinium, blockers of these channels [15–17], eliminated the hyperpolarizing effect of lactate, which depends on the presence of extracellular Ca^{2+}, it is possible that cell swelling activates these channels allowing an intracellular Ca^{2+} influx and subsequent opening of Ca^{2+}-dependent K^+ channels being partly responsible for lactate's hyperpolarizing effect.

Previous studies had shown that lactate metabolism in the perfused rat liver leads to an increase in intracellular pH [36]. This favors activation of Ca^{2+}-dependent K^+ channels [14,37,38] and hence hyperpolarization of the cell membrane. This is also reflected by the antagonistic effect of amiloride (1 mmol/l), a blocker of the Na^+/H^+-antiporter of the cell membrane which leads to cell acidification, on the lactate induced hyperpolarization.

The physiological relevance of the observed effects

needs to be investigated in further studies because relatively high lactate (20 mmol/l) concentrations were necessary to hyperpolarize the hepatocyte membrane using physiological extracellular Ca^{2+} concentrations. Lower substrate concentrations necessitated a higher extracellular Ca^{2+} (2.56 mmol/l) concentration. The question remains open, however, if this also applies under in vivo conditions because non-selective cation channels can be activated by certain hormones [30] which could allow an increase in intracellular Ca^{2+} to the pertinent level by lactate at a lower Ca^{2+} concentration in the extracellular space. Moreover, it is possible that under certain pathological conditions, the observed effects might gain in importance. It has been shown that the presence of certain tumors can be associated with increased blood lactate levels because the biochemical energy of tumors is mainly produced via anaerobic metabolism [37,39,40]. Further, increased plasma Ca^{2+} levels in subjects with certain tumors have also been reported (malignancy-associated hypercalcemia [41]). It remains to be clarified if these effects contribute to the syndrome of cancer anorexia [39], because the hepatic membrane potential has been suggested to play a role in the control of feeding [9], and because lactate has been observed to reduce food intake in rats by acting on the liver [6,7].

In summary, in the present study, we have shown that the mechanisms underlying the hyperpolarization of the liver cell membrane induced by lactate, pyruvate, and fructose are similar to those underlying hyperpolarization in hypo-osmotic medium. The hyperpolarization of the hepatocyte membrane induced by superfusion with these substrates therefore seems to be due to cell swelling and appears to be linked to a stimulation of gluconeogenesis. It may be brought about by an influx of Ca^{2+} into hepatocytes via non-selective cation channels coupled to an opening of K^+ channels and an activation of the Na^+/K^+-ATPase. Increased Na^+ and Cl^- efflux from the hepatocytes, caused by the activation of the Na^+/K^+-ATPase or triggered by the hyperpolarization, could partly counteract the substrate-induced cell swelling.

References

[1] N. Friedmann, G. Dambach, Antagonistic effect of insulin on glucagon-evoked hyperpolarization. A correlation between changes in membrane potential and gluconeogenesis, Biochim. Biophys. Acta 596 (1980) 180–185.

[2] G. Dambach, N. Friedmann, Substrate-induced membrane potential changes in the perfused rat liver, Biochim. Biophys. Acta 367 (1974) 366–370.

[3] R. Rossi, E. Scharrer, Hyperpolarization of the liver cell membrane by palmitate as affected by glucose and lactate – implications for control of feeding, J. Auton. Nerv. Syst. 56 (1995) 45–49.

[4] S.K. Moule, J.D. McGivan, Regulation of the plasma membrane potential in hepatocytes – mechanism and physiological significance, Biochim. Biophys. Acta 1031 (1990) 383–397.

[5] M.I. Friedman, Hepatic-cerebral interactions in insulin-induced eating and gastric acid secretion, Brain Res. Bull. 5 (1980) 63–68.

[6] W. Langhans, G. Egli, E. Scharrer, Selective hepatic vagotomy eliminates the hypophagic effect of different metabolites, J. Auton. Nerv. Syst. 13 (1985) 255–262.

[7] H. Nagase, G.A. Bray, D.A. York, Effects of pyruvate and lactate on food intake in rat strains sensitive and resistant to dietary obesity, Physiol. Behav. 59 (1996) 555–560.

[8] M.G. Tordoff, M.I. Friedman, Hepatic control of feeding: effect of glucose, fructose and mannitol infusion, Am. J. Physiol. 254 (1988) R969–R976.

[9] M. Russek, Current status of the hepatostatic theory of food intake, Appetite 2 (1981) 137–143; 157–162.

[10] R. Wondergem, L.B. Castillo, Quinine decreases hepatocyte transmembrane potential and inhibits amino acid transport, Am. J. Physiol. 254 (1988) G795–G801.

[11] A.P. Halestrap, R.M. Denton, Specific inhibition of pyruvate transport in rat liver mitochondria and human erythrocytes by α-cyano-d-hydroxycinnamate, Biochem. J. 138 (1974) 313–316.

[12] R.B. Gregory, M.N. Berry, The characterization of perfluorosuccinate as an inhibitor of gluconeogenesis in isolated rat hepatocytes, Biochem. Pharmacol. 38 (1989) 2867–2872.

[13] G.M. Burgess, M. Claret, D.H. Jenkinson, Effects of quinine and apamin on the calcium-dependent potassium permeability of mammalian hepatocytes and red cells, J. Physiol. (Lond.) 317 (1981) 67–90.

[14] N.S. Cook, The pharmacology of potassium channels and their therapeutic potential, Trends Pharmacol. Sci. 9 (1988) 21–28.

[15] P. Poronnik, M.C. Ward, D.I. Cook, Intracellular Ca^{2+} release by flufenamic acid and other blockers of non-selective cation channels, FEBS Lett. 296 (1992) 245–248.

[16] X.-C. Yang, F. Sachs, Block of stretch-activated ion channels in *Xenopus* oocytes by gadolinium and calcium ions, Science 243 (1989) 1068–1071.

[17] D. Filipovic, H. Sackin, A calcium-permeable stretch-activated cation channel in renal proximal tubule, Am. J. Physiol. 260 (1991) F119–F129.

[18] O. Thastrup, P.J. Cullen, B.K. Drobak, M.R. Hanley, A.P. Dawson, Thapsigargin, a tumor promoter, discharges intra-

cellular Ca^{2+} stores by specific inhibition of the endoplasmic reticulum Ca^{2+}-ATPase, Proc. Natl. Acad. Sci. USA 87 (1990) 2466–2470.
[19] S.-M. Lee, M.G. Clemens, Subacinar distribution of hepatocyte membrane potential response to stimulation of gluconeogenesis, Am. J. Physiol. 263 (1992) G319–G326.
[20] L.D. Howard, R. Wondergem, Effects of anisosmotic medium on cell volume, transmembrane potential and intracellular K^+ activity in mouse hepatocytes, J. Membr. Biol. 100 (1987) 53–61.
[21] P. Fafournoux, C. Demigne, C. Remesy, Carrier-mediated uptake of lactate in rat hepatocytes. Effects of pH and possible mechanisms for L-lactate transport, J. Biol. Chem. 260 (1985) 292–299.
[22] L. Sacca, R. Hendler, R.S. Sherwin, Hyperglycemia inhibits glucose production in man independent of changes in glucoregulatory hormones, J. Clin. Endocrinol. Metab. 47 (1978) 1160–1163.
[23] R. Rognstad, The control of gluconeogenesis in the liver by glucose, in: N. Kraus-Friedmann (Ed.), Hormonal Control of Gluconeogenesis, Vol. III, CRC Press, Boca Raton, FL, 1986, pp. 249–256.
[24] J.E. Feliu, L. Hue, H.G. Hers, Hormonal control of pyruvate kinase activity and of gluconeogenesis in isolated hepatocytes, Proc. Natl. Acad. Sci. USA 73 (1976) 2762–2766.
[25] C. Sutherland, R.M. O'Brien, D.K. Granner, New connections in the regulation of PEPCK gene expression by insulin, Phil Trans. R. Soc. Lond. B 351 (1996) 191–199.
[26] A. Gasbarrini, A.B. Borle, H. Farghali, C. Bender, A. Francavilla, D. VanThiel, Effect of anoxia on intracellular ATP, Na_i^+, Ca_i^{2+}, Mg_i^{2+}, and cytotoxicity in rat hepatocytes, J. Biol. Chem. 267 (1992) 6654–6663.
[27] E. Scharrer, R. Rossi, D.A. Sutter, M.-C. Seebacher, S. Boutellier, T.A. Lutz, Hyperpolarization of hepatocytes by 2,5-anhydro-D-mannitol (2,5-AM): implications for hepatic control of food intake, Am. J. Physiol. 272 (1997) R874–R878.
[28] H. Huang, H. St. Jean, M.J. Coady, J.-Y. Lapointe, Evidence for coupling between Na^+ pump activity and TEA-sensitive K^+ currents in *Xenopus laevis* oocytes, J. Membr. Biol. 143 (1995) 29–35.
[29] K. Wang, R. Wondergem, Hepatocyte water volume and potassium activity during hypotonic stress, J. Membr. Biol. 135 (1993) 137–144.
[30] J. Graf, D. Häussinger, Ion transport in hepatocytes: mechanisms and correlations to cell volume, hormone actions and metabolism, J. Hepatol. 24, (Suppl. 1) (1996) 53–77.
[31] C.A. Sandford, J.H. Sweiry, D.H. Jenkinson, Properties of a cell volume-sensitive potassium conductance in isolated guinea-pig and rat hepatocytes, J. Physiol. (Lond.) 447 (1992) 133–148.
[32] W.E. Khalbuss, R. Wondergem, Involvement of cell calcium and transmembrane potential in control of hepatocyte volume, Hepatology 13 (1991) 962–969.
[33] A. Galvez, G. Gimenez-Gallego, J.P. Reuben, L. Roy-Contancin, P. Feigenbaum, G.J. Kaczorowski, M.L. Garcia, Purification and characterization of a unique, potent, peptidyl probe for the high conductance calcium-activated potassium channel from venom of the scorpion *Buthus tamulus*, J. Biol. Chem. 265 (1990) 11083–11090.
[34] P. Christophersen, B. Vestergaard-Bogind, Cetiedil, a common inhibitor of the Ca-activated K^+-channel and Ca-pump in human erythrocytes, Acta Physiol. Scand. 124, (Suppl. 542) (1985) 167.
[35] C.E. Bear, A nonselective cation channel in rat liver cells is activated by membrane stretch, Am. J. Physiol. 258 (1990) C421–C428.
[36] R.D. Cohen, R.A. Iles, D. Barnett, M.E.O. Howell, J. Strunin, The effect of changes in lactate uptake on the intracellular pH of the perfused rat liver, Clin. Sci. 41 (1971) 159–170.
[37] J.M. Arbeit, M.E. Burt, L.V. Rubinstein, C.M. Gorschboth, M.F. Brennan, Glucose metabolism and the percentage of glucose derived from alanine: response to exogenous glucose infusion in tumor-bearing and non-tumor-bearing rats, Cancer Res. 42 (1982) 4936–4942.
[38] D.A. Klaerke, H. Wiener, T. Zeuthen, P.L. Jorgensen, Ca^{2+} activation and pH dependence of a maxi K^+ channel from rabbit distal colon epithelium, J. Membr. Biol. 136 (1993) 9–21.
[39] E. Cersosimo, P.W.T. Pisters, G. Pesola, A. Rogatko, N.A. Vydelingum, D. Bajorunas, M.F. Brennan, The effect of graded doses of insulin on peripheral glucose uptake and lactate release in cancer cachexia, Surgery 109 (1991) 459–467.
[40] Y.-L. Dong, D. Fleming, K.F. Huang, D.N. Herndon, T.Z. Yan, J.P. Waymack, Effect of insulin-like growth factor 1 on host response to tumor, J. Surg. Oncol. 53 (1993) 121–127.
[41] D.J. Teachout, S.M. Taylor, F.J. Archer, A comparison of serum ionized calcium concentrations and serum total calcium concentrations in dogs with lymphoma, J. Vet. Med. A44 (1997) 195–200.