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Effect of 2,5-anhydro-D-mannitol on membrane potential in rat hepatocyte couplets and hepatocyte monolayer cultures

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Abstract

The fructose analogue 2,5-anhydro-D-mannitol (2,5-AM), which depletes liver cells of ATP, has been shown to alter liver cell membrane potential (V_m) in situ and in superfused liver slices. To study this effect of 2,5-AM on hepatocytes in more detail, patch-clamp experiments in the current-clamp mode were performed using two established models, rat hepatocyte couplets and confluent rat hepatocytes in primary culture. 2,5-AM, which has previously been shown to hyperpolarize hepatocytes in superfused liver slices and in vivo, failed to alter V_m of hepatocyte couplets. Increasing intracellular Ca^{2+} by addition of thapsigargin or ionomycin also did not evoke a change of V_m. This is most likely due to a lack of Ca^{2+}-dependent K^+ channels in rat hepatocyte couplets. In contrast, 2,5-AM depolarized the cells in confluent hepatocyte monolayers. This depolarization was mimicked after inhibition of Na^+/K^+ ATPase by ouabain. Ouabain was also able to block 2,5-AM's effect on monolayer V_m. Thus, 2,5-AM affects the membrane potential of isolated and cultured hepatocytes in a way not comparable with cells integrated in the liver.

Keywords: 2,5-Anhydro-D-mannitol; Membrane potential; Hepatocyte couplet; Hepatocyte monolayer

1. Introduction

It has been postulated that the membrane potential (V_m) of hepatocytes plays a significant role in the control of food intake [1]. According to Russek, hepatocyte V_m determines the discharge rate of vagal afferents, leading in the CNS to a satiety or hunger signal, respectively [1]. It was recently shown by our group that the fructose analogue 2,5-anhydro-D-mannitol (2,5-AM), a drug that increases food intake via a liver-dependent mechanism [2], hyperpolarized V_m of hepatocytes in superfused liver slices from rats and mice, probably by activation of Ca^{2+}-dependent K^+ channels [3]. This finding was unexpected with regard to Russek's theory [1]. In contrast to fructose, which also hyperpolarizes hepatocyte V_m [4,5], the Na^+/K^+ ATPase is not involved in the V_m increase induced by 2,5-AM [3]. The same effect of 2,5-AM on hepatocyte V_m could be confirmed in vivo [6]. Strategies to increase $[Ca^{2+}]_i$, e.g., by inhibiting endoplasmic Ca^{2+} ATPase by thapsigargin, led to a hyperpolarization of liver slices as well [5].

2,5-AM inhibits gluconeogenesis and glycogenolysis in rat hepatocytes [7–10]. Hepatocytes convert 2,5-AM via 2,5-anhydromannitol-1-phosphate (2,5-AM-1-P) to 2,5-anhydromannitol-1,6-bisphosphate (2,5-AM-1,6-P_2), which cannot be further metabolized. Whereas 2,5-AM-1-P decreases glycogenolysis

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by inhibiting liver glycogen phosphorylase, the 1,6-bisphosphate blocks fructose 1,6-bisphosphatase and stimulates pyruvate kinase, thereby leading to inhibition of gluconeogenesis [8,10]. Phosphorylation of 2,5-AM traps hepatic phosphate and decreases liver ATP in vivo [11–13]. 2,5-AM also decreases ATP content in isolated rat hepatocytes [9].

States where hepatic ATP content is decreased, e.g., anoxia, lead to a rise in intracellular Ca^{2+} ($[Ca^{2+}]_i$) [14,15]. The reduced ATP levels or the change in the ratio of ATP to ADP are the cause for Ca^{2+} release from intracellular stores [15].

The present study was undertaken to clarify the mechanisms involved in the hyperpolarizing effects of 2,5-AM in liver cells. The experiments were performed with the slow whole-cell patch-clamp technique using two different hepatocyte models: rat hepatocyte couplets and confluent rat hepatocytes in primary culture. Parts of this study have been presented previously in abstract form [16].

2. Materials and methods

2.1. Solutions

The standard buffer used for cell isolation and the experiments was a Krebs–Henseleit solution (KH), which contained (in mmol/l): NaCl 120; KCl 4.8, KH_2PO_4 1.2, $NaHCO_3$ 24, $CaCl_2$ 1.3, $MgCl_2$ 1, gassed with 95% O_2/5% CO_2 at 37°C. For the isolation procedure different factors were added or omitted (see below). Solutions had an osmolarity of 290 ± 2 mosmol/l. pH was always adjusted to 7.4 and checked after addition of the substrates. The pipette solution contained (in mmol/l): K^+-gluconate 95; KCl 30; NaH_2PO_4 1.2; Na_2HPO_4 4.8; D-glucose 5; Ca^{2+}-gluconate 0.73; EGTA 1; $MgCl_2$ 1.03; ATP 1; pH was adjusted to 7.2. Immediately before filling the pipettes, amphotericin B was added at a concentration of 100 mg/l and the solution was ultrasonicated.

2.2. Hepatocyte isolation

Rat hepatocytes were isolated following the methods of Berry and Friend [17] and Seglen [18], with slight modifications. Male rats (ZUR:SD, Labortierkunde, University of Zurich) weighing 200–300 g were anesthetized with pentobarbitone (50 mg/kg) and the abdominal cavity opened. The liver was perfused via the portal vein in situ with a Ca^{2+}-free KH solution (containing 5 mmol/l glucose) at a rate of 24 ml/min by means of a peristaltic pump. During perfusion animals died through bleeding. After 8–10 min the solution was changed to a KH buffer containing 1.3 mmol/l $CaCl_2$, 1% bovine serum albumin, and 0.04% collagenase. Collagenase perfusion was maintained for 5–6 min; thereafter, the liver was removed carefully into an ice cold KH solution (containing $CaCl_2$ and 0.2% bovine serum albumin, but without collagenase and glucose). The liver capsule was removed and the tissue carefully minced with forceps. The cell suspension was filtered through a nylon mesh (mesh size 60 μm) and centrifuged twice with 25 g for 5 min. Trypan blue exclusion was $>85\%$. The harvested cells were used in two different ways.

2.3. Hepatocyte couplets

For hepatocyte couplets an aliquot of around 300 000 cells was diluted in 10 ml of Leibovitz-15 tissue culture medium (supplemented with 2 mmol/l glutamine, penicillin–streptomycin (100 U/ml: 100 μg/ml), and 10% fetal calf serum) and plated on glass coverslips. Among the isolated cells, a varying amount of approximately 10–20% were cell pairs (couplets). The hepatocytes were incubated in a humidified air atmosphere chamber at 37°C for 4–8 h. During this incubation time, the couplets retained their polarity and formed canalicular spaces between them [19,20]. For the experiments, coverslips were transferred to a measuring chamber 4 to maximal 8 h after cell isolation. Only couplets with a visible canalicular space were patched or impaled, respectively.

2.4. Hepatocyte monolayers

Monolayers were cultivated by the method of Wehner and Guth [21]. The isolated hepatocytes were plated at a higher density (10^6 cells/ml) in KH buffer on collagen-coated Petriperm dishes (H. Sauer, Reutlingen, Germany). After 20 min incubation in an atmosphere of 5% CO_2/95% air at 37°C,

unattached cells were washed off and the KH buffer replaced by Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mmol/l glutamine, penicillin–streptomycin (100 U/ml: 100 μg/ml), 10% fetal calf serum, 10^{-6} mol/l dexamethasone, 10^{-8} mol/l T_3/T_4, and 5 μg/l bovine insulin. Cells were incubated in 5% CO_2/95% air at 37°C. Hepatocytes became confluent within 1 day and formed canaliculi at their adjacent membrane areas. The medium was exchanged daily. The monolayers were used for the experiments 1–3 days after cell isolation.

2.5. Patch-clamp recordings

The measuring chamber (volume 0.5 ml) was continuously perfused at a rate of 5–10 ml/min with KH buffer. In some experiments, Ca^{2+} content was increased as indicated in the text. The experiments were performed at 37°C. Pipettes were made from borosilicate capillaries (Jencons Scientific, Bedfordshire, UK, o.d. 2 mm, i.d. 1 mm) with a final input resistance of 5–10 MΩ. The data were obtained using the slow whole-cell patch-clamp technique [22,23] with amphotericin B (100 mg/l) in the pipette solution. V_m was recorded in the zero current-clamp mode. Membrane conductances (G_m) were obtained by clamping the voltage to ±60 mV for 100 ms from the spontaneous V_m value. From the resulting currents, conductance values were calculated after subtracting the respective series resistance. Data were sampled using a patch-clamp amplifier (RK-400, Biologics, Meylan, France) and V_m values recorded on a pen recorder. After obtaining a Gigaseal, experiments were started when a stable membrane potential was reached.

2.6. Intracellular recording of V_m

Pipettes were drawn in a horizontal puller (Sachs–Fleming Micropipette Puller PC-84; Sutter, San Raphael, CA, USA) from glass capillaries (Science Products, Hofheim, Germany, o.d. 1.5 mm, i.d. 1.05 mm) and filled with 0.5 mol/l KCl. In KH solution, the pipettes had an input resistance of 70–125 MΩ. Pipettes were mounted on the head stage of the patch-clamp amplifier and impaled into the hepatocytes in the measuring chamber under visual control. V_m was recorded in the zero current-clamp mode.

2.7. Biochemicals

All standard chemicals and hormones for cell culture were obtained from Fluka or Sigma, Buchs, Switzerland. 2,5-Anhydro-D-mannitol was from Toronto Research Chemicals, North York, Canada. Cell culture media, glutamine and penicillin–streptomycin were purchased from Biochrom, Berlin, Germany.

2.8. Statistics

Data are presented as mean ± S.E. with the number of experiments indicated in parentheses. Statistical significance of the effects was tested by comparing the value under the drug with the mean of the control values before drug addition and after drug removal (paired two-sided Student's *t*-test). A P value of less than 0.05 was considered significant.

3. Results

3.1. Hepatocyte couplets

Intracellular recording of V_m in eight hepatocyte couplets yielded a value of -27.3 ± 3.0 mV under basal conditions. The application of the K^+ channel blocker Ba^{2+} at a concentration of 5 mmol/l depolarized V_m reversibly by 10.4 ± 2.2 mV ($n = 8$), something which proved the presence of a basal K^+ conductance in these cells.

The fructose analogue 2,5-AM was shown to hyperpolarize rat liver cells 5–9 min after its infusion into the portal vein in vivo [6]. Therefore, hepatocyte couplets were superfused with 2,5-AM for at least 8 min to allow the substance to act on the cells. A concentration of 2.5 mmol/l was chosen as it proved to be effective in causing a hyperpolarization in superfused rat and mouse liver slices [3]. However, 2,5-AM failed to show an effect on membrane potential ($\Delta V_m = 1.0 \pm 0.5$ mV, $n = 7$; $P > 0.05$).

All of the following experiments were performed in the slow whole-cell recording mode of the patch-clamp technique due to its advantages over conventional electrophysiology [23]. In 58 hepatocyte couplets, V_m was -36.5 ± 1.6 mV under basal conditions, which was significantly higher than in intracellular electrode measurements ($P < 0.05$). Ba^{2+}

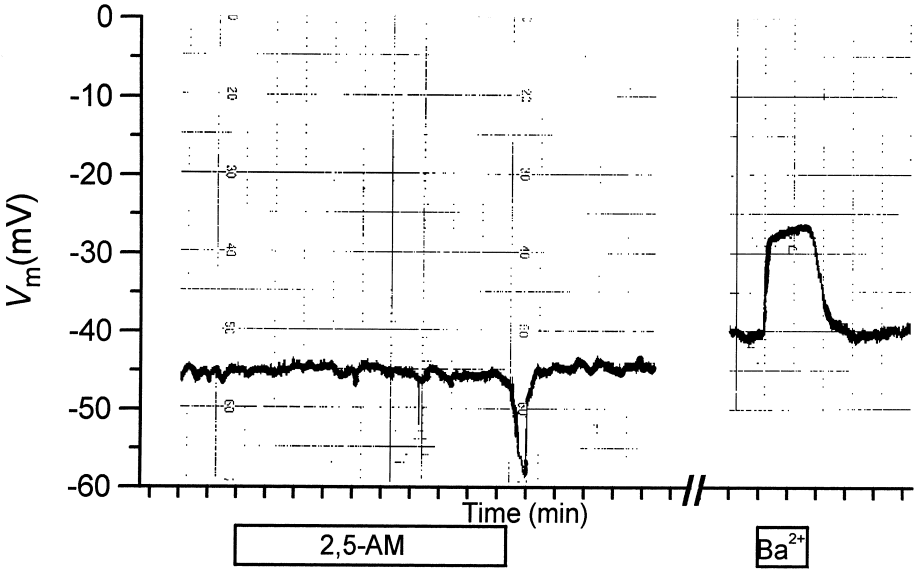

Fig. 1. Original recording of the membrane voltage (V_m) of a rat hepatocyte couplet. The ticks on the abscissa mark time intervals of 1 min each. 2,5-AM (2.5 mmol/l) and Ba^{2+} (5 mmol/l) were present at the times indicated. The Ba^{2+} effect was recorded after a time gap. The voltage deflection at the end of the 2,5-AM superfusion period is an artifact due to the solution exchange in this particular experiment.

(5 mmol/l) depolarized V_m reversibly by 8.6 ± 0.9 mV in 17 couplets (Fig. 1 and 2). The K^+ channel blocker decreased the membrane conductance (G_m) by 36% from 19.4 ± 3.3 nS to 12.4 ± 1.5 nS ($n = 14$).

Again, 2.5 mmol/l 2,5-AM failed to show an effect on membrane potential (Fig. 1), V_m in nine couplets changed by -1.1 ± 1.3 mV ($P > 0.05$) (Fig. 2). Because a buffer concentration of 2.6 mmol Ca^{2+}/l was used in an earlier study with liver slices [3], measurements were repeated with 2.6 mmol/l Ca^{2+} present. Again, 2.5 mmol/l 2,5-AM failed to evoke a change in V_m of couplets (Fig. 3).

The isolation procedure of the cells might have changed the activities of their metabolic enzymes,

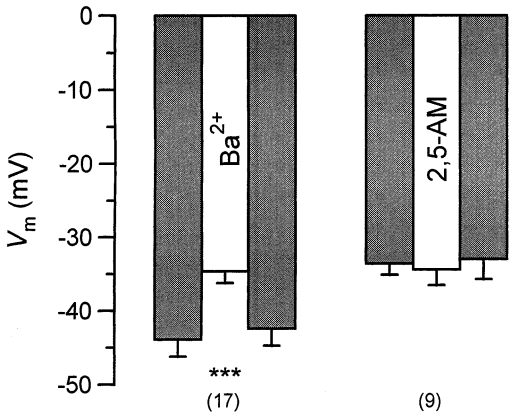

Fig. 2. Effects of Ba^{2+} (5 mmol/l) and of 2,5-AM (2.5 mmol/l) on membrane voltage (V_m) of rat hepatocyte couplets. Numbers in parentheses indicate the number of experiments. ***$P < 0.001$ versus the mean of the control values (dark bars) before addition and after removal of the respective substance.

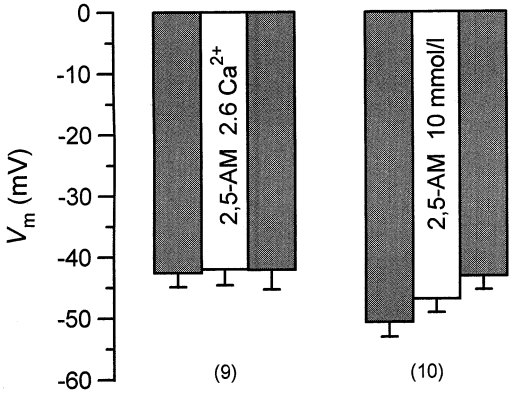

Fig. 3. No effect of 2.5 mmol/l 2,5-AM in a buffer solution containing 2.6 mmol/l Ca^{2+} or of 10 mmol/l 2,5-AM in standard solution on membrane voltage (V_m) of rat hepatocyte couplets. Numbers in parentheses indicate the number of experiments. Dark bars represent V_m values before addition and after removal of the substance, respectively.

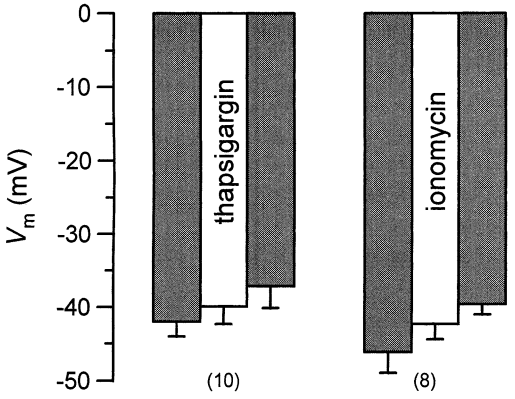

Fig. 4. No effect of thapsigargin (500 nmol/l) or ionomycin (1 μmol/l) on membrane voltage (V_m) of rat hepatocyte couplets. Numbers in parentheses indicate the number of experiments. Dark bars represent V_m values before addition and after removal of the substance, respectively.

thus decreasing the level of 2,5-AM phosphorylation. To get around this possible obstacle of the preparation used, 2,5-AM was added at a higher concentration. However, even at a concentration of 10 mmol/l, the fructose analogue did not influence couplet V_m in the time course of 8–10 min ($\Delta V_m = 1.1 \pm 1.4$ mV; $n = 10$) (Fig. 3). Changes in membrane conductance could not be detected at any 2,5-AM concentration used.

The mentioned hyperpolarization induced by 2,5-AM in superfused liver slices was blocked by a selective inhibitor of small conductance Ca^{2+}-dependent K^+ channels, apamin [3]. Inhibition of endoplasmic Ca^{2+} ATPase by thapsigargin also caused a hyperpolarization in the same preparation [5]. These results suggest that 2,5-AM increases $[Ca^{2+}]_i$ and opens Ca^{2+}-dependent K^+ channels to exert its effect on V_m in hepatocytes. To obtain direct information about these channels in our preparation, $[Ca^{2+}]_i$ was raised by two means.

Firstly, cytosolic Ca^{2+} concentration was increased by blocking endoplasmic Ca^{2+} ATPase by thapsigargin. This drug was shown to increase $[Ca^{2+}]_i$ within 1 min from intracellular stores in isolated rat hepatocytes [24,25]. The application of thapsigargin (500 nmol/l) during 8–10 min failed to effect V_m in hepatocyte couplets ($\Delta V_m = 0.3 \pm 1.0$ mV; $n = 10$) (Fig. 4).

Secondly, Ca^{2+} influx from the extracellular solution was enhanced by addition of the Ca^{2+} ionophore ionomycin. Addition of 1 μmol/l of this ionophore to the superfusion buffer for at least 8 min also did not change basal V_m in rat hepatocyte couplets ($\Delta V_m = 1.7 \pm 0.8$ mV; $n = 8$) (Fig. 4).

The results from the two latter treatments suggest

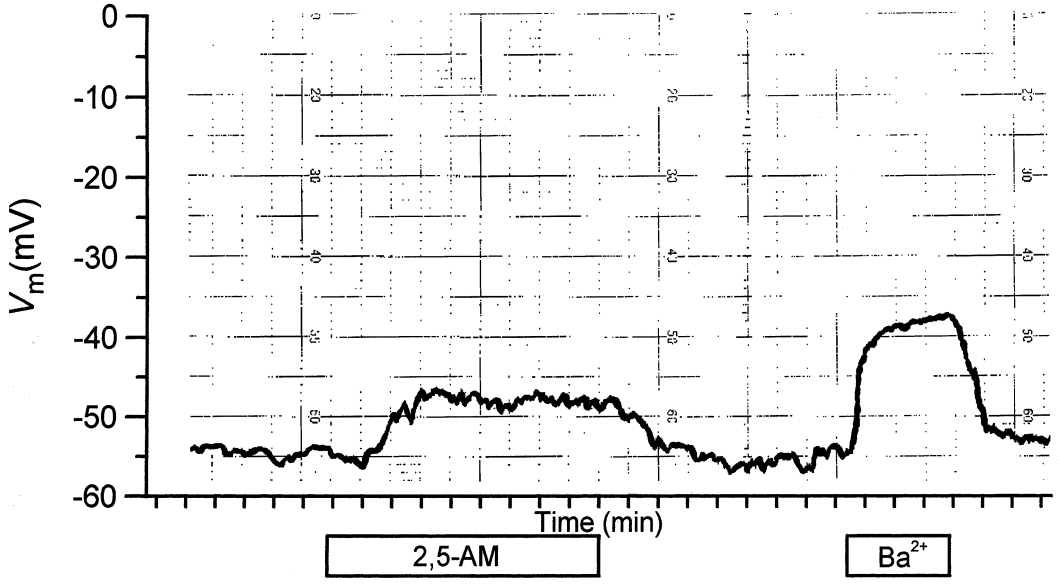

Fig. 5. Original recording of the membrane voltage (V_m) of a rat hepatocyte in primary monolayer culture. The ticks on the abscissa mark time intervals of 1 min each. 2,5-AM (2.5 mmol/l) and Ba^{2+} (5 mmol/l) were present at the times indicated.

that the cells in this preparation are lacking Ca^{2+}-dependent K^+ channels. Otherwise, the hepatocytes should have hyperpolarized after the induced $[Ca^{2+}]_i$ increase. As it is known from cultured epithelial cell lines, single cells used after isolation may show different membrane conductances from native tissue and regain properties of the original epithelium only after forming confluent monolayers [26,27]. Therefore, we used confluent hepatocyte monolayers to investigate the effect of 2,5-AM.

3.2. Confluent monolayers

In confluent monolayers, basal V_m was significantly higher than in hepatocyte couplets with a value of -43.1 ± 1.0 mV ($n = 58$; $P < 0.001$). Conductance measurements could not be performed due to the intense electrical coupling between these cells. Similar to couplets, 5 mmol/l Ba^{2+} decreased V_m by 11.1 ± 0.4 mV ($n = 29$) (Figs. 5 and 6). The addition of 2,5-AM at a concentration of 2.5 mmol/l induced a reversible depolarization of hepatocyte V_m by 7.1 ± 0.7 mV ($n = 15$) (Figs. 5 and 6). The Na^+/K^+ ATPase inhibitor ouabain (1 mmol/l) depolarized monolayers by 5.5 ± 0.7 mV ($n = 11$) similar to 2,5-AM (Fig. 7).

Because 2,5-AM reduces the ATP content of hepatocytes by trapping phosphate [9,11–13], this might have been the cause of an inhibition of Na^+/K^+ ATPase. Indeed, when 2,5-AM was applied in the presence of ouabain, a further depolarization induced by the fructose analogue was absent (Fig. 7).

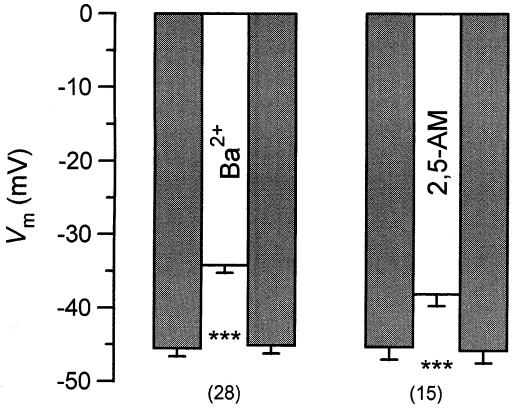

Fig. 6. Effects of Ba^{2+} (5 mmol/l) and 2,5-AM (2.5 mmol/l) on membrane voltage (V_m) of rat hepatocytes in primary monolayer culture. Numbers in parentheses indicate the number of experiments. ***$P < 0.001$ versus the mean of the control values (dark bars) before addition and after removal of the respective substance.

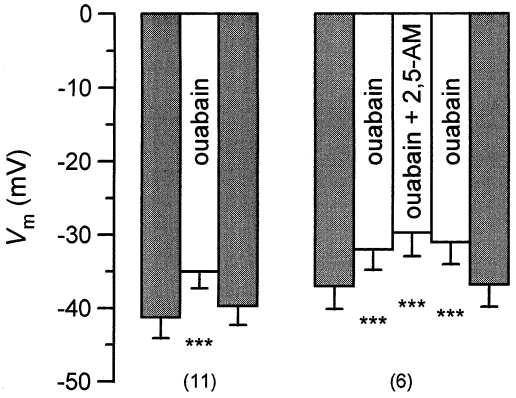

Fig. 7. Effects of ouabain (1 mmol/l) and of the combined effects of ouabain and 2,5-AM (2.5 mmol/l) on membrane voltage (V_m) of rat hepatocytes in primary monolayer culture. Numbers in parentheses indicate the number of experiments. ***$P < 0.001$ versus the mean of the control values (dark bars) before addition and after removal of the respective substances. V_m under 2,5-AM and ouabain is not statistically different from V_m under ouabain alone.

4. Discussion

The monosaccharide fructose was shown to hyperpolarize V_m of hepatocytes in superfused mouse or rat liver slices dependent on the Na^+/K^+ ATPase; a Ca^{2+}-dependent activation of K^+ channels was also involved in this hyperpolarization [4,5]. This effect could be mimicked by cell swelling induced by hypo-osmotic medium and was likely due to gluconeogenesis. Fructose is metabolized to glucose which increases intracellular osmolarity leading to water influx and cell swelling, thereby hyperpolarizing the cells [5].

In contrast, the fructose analogue 2,5-AM traps phosphate in the liver by getting phosphorylated to the non-metabolizable 2,5-AM-1,6-P_2, thereby inhibiting gluconeogenesis and glycogenolysis [8–11]. This diminishes the ATP level of liver cells in vivo and also of isolated hepatocytes [9,11–13].

As it is known that a decrease of ATP content increases $[Ca^{2+}]_i$ in isolated hepatocytes and stimulates Ca^{2+} release from the perfused liver [14,15], one would expect that 2,5-AM exerts the same effect on

$[Ca^{2+}]_i$. The fructose analogue causes a hyperpolarization of liver cells in vivo [6] and also in superfused liver slices [3]. In the latter preparation, this hyperpolarization could be blocked by apamin, an inhibitor of small conductance Ca^{2+}-dependent K^+ channels. Thapsigargin, which increases $[Ca^{2+}]_i$ by blocking endoplasmic Ca^{2+} ATPase [24,25], also hyperpolarized liver slices [5]. Taken together, these data suggest that 2,5-AM increases $[Ca^{2+}]_i$ by decreasing ATP levels. The rise in $[Ca^{2+}]_i$ would cause the activation of Ca^{2+}-dependent K^+ channels resulting in the observed hyperpolarization.

To test this hypothesis and study the putative Ca^{2+}-dependent K^+ channels in more detail, a model suitable for patch clamping was chosen: the isolated rat hepatocyte couplet. Couplets retain their polarity and secrete into a canalicular space formed between them only a few hours after their isolation [19,20]. Therefore, secreting couplets are a model representing the smallest functioning liver unit.

In contrast to its effect in the intact liver or in superfused liver slices, 2,5-AM failed to change V_m in couplets. This was true for conventional electrophysiology as well as for slow whole-cell recordings. Whereas the membrane potential measured with intracellular microelectrodes was similar to the one recorded in superfused liver slices and liver cells in vivo [3,6], V_m recordings with the slow whole-cell technique yielded higher values. This was probably due to smaller leak currents compared with intracellular microelectrode measurements [23].

It cannot be excluded that the activity of metabolic enzymes was changed in our preparation. However, it was shown by different groups that 2,5-AM is effective in isolated hepatocytes in the same concentration range used by us, including its effect on intracellular ATP [8–10]. Increasing the concentration of the fructose analogue, though, could not alter its inefficiency in the couplets. Means to directly increase $[Ca^{2+}]_i$ by thapsigargin or ionomycin also failed to show an effect on V_m. This can only be explained by a lack of Ca^{2+}-dependent K^+ channels, making this model unresponsive for the parameters investigated.

Our finding confirms the studies of other groups, who found a Ca^{2+}-dependent K^+ conductance only in isolated hepatocytes from guinea pigs and rabbits, but never in those from rats [28–31]. Only one report stated the existence of such channels in isolated rat hepatocytes [32], but this was never confirmed by other groups. Accordingly, Ca^{2+} ionophores like ionomycin or A23187 were reported to be ineffective influencing V_m or K^+ conductance in cells obtained from rats [30,33]. Thapsigargin was also ineffective in our study, in contrast to its action in mouse liver slices [5].

Epithelial cells may loose some of their characteristic properties like specific ion conductances after isolation, unless they are grown to confluence and form polarized monolayers [26,27]. Although hepatocyte couplets are polarized and represent a functional secretory liver unit [19,20], we also decided to use confluent hepatocyte monolayers to investigate a possible 2,5-AM effect.

In contrast to our expectations, 2,5-AM depolarized hepatocyte monolayers. The fructose analogue decreases intracellular ATP and this could diminish Na^+/K^+ ATPase activity. Ouabain depolarized the monolayers in a similar fashion and prevented the depolarization induced by 2,5-AM. This suggests that 2,5-AM has an impact on Na^+/K^+ ATPase activity, probably by decreasing ATP levels. However, this was not further investigated, as 2,5-AM did not alter monolayer V_m as it does in liver slices or in the intact organ in vivo.

The reason for the discrepancies between hepatocyte couplets and confluent monolayers are currently unknown. Furthermore, at present we do not know why these two models behave differently from hepatocytes in the intact tissue. In vivo as well as in liver slices, non-hepatocyte liver cells, like endothelial, stellate and Kupffer cells, are present, which might influence the expression of membrane proteins in hepatocytes. When these cells – or specific factors released by them – are missing, rat hepatocytes might not be able to express Ca^{2+}-dependent K^+ channels. This could be a possible explanation why the two hepatocyte models investigated by us were unable to respond with a V_m increase to 2,5-AM. However, as hepatocytes isolated from guinea pigs or rabbits by the same isolation procedure express Ca^{2+}-dependent K^+ channels [28,30,31], this seems to be distinctly species specific.

In conclusion, 2,5-AM failed to influence hepatocyte V_m in the same way as reported in earlier studies using liver cells in intact tissue and in vivo. The lack

of hyperpolarization is most likely due to a loss of certain K^+ channels, at least in hepatocyte couplets. At this point, it cannot be excluded that other functions are also altered in isolated rat hepatocytes.

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