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Volume-regulatory K^+ efflux during concentrative uptake of alanine in isolated rat hepatocytes

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Changes in cell volume and $^{42}K^+$ efflux associated with concentrative alanine uptake were studied in isolated rat hepatocytes suspended in Krebs–Ringer bicarbonate buffer. After addition of 10mM-alanine, cellular water volume increased by 15% and the rate constant of $^{42}K^+$ efflux by 250%. Alanine-induced $^{42}K^+$ efflux was abolished by quinine and was strongly decreased when the cell-volume increase was counteracted by sucrose. The results suggest that K^+ efflux during alanine uptake is implicated in a volume-regulatory response.

We have previously presented evidence that Na^+–alanine co-transport in rat hepatocytes is accompanied by an increase in the K^+ permeability of the cell membrane (Kristensen, 1980). Subsequently, a similar phenomenon has been reported in epithelial cells, where the basolateral cell membrane becomes more permeable to K^+ during coupled influx of Na^+ and amino acids or sugars across the luminal cell membrane (Gunter-Smith *et al.*, 1982; Grasset *et al.*, 1983). Coupled influx of Na^+ and a non-electrolyte tends to increase the cytosolic concentration of the transported non-electrolyte, to increase the cytosolic Na^+ concentration, and possibly to increase the cytosolic K^+ concentration through stimulation of the Na^+–K^+ pump. Moreover, Na^+-coupled influx of neutral solutes will tend to depolarize the cell membrane (Rose & Schultz, 1971; Folke & Paloheimo, 1975). An increase in the K^+ permeability could serve as a homoeostatic mechanism towards such challenges. Increased K^+ permeability may prevent excessive cellular K^+ accumulation or even lead to loss of cellular K^+ with accompanying Cl^- and water, thereby contributing to regulation of the cell volume. In addition, increased K^+ permeability may counteract the tendency to depolarization and thereby contribute to maintain the Na^+ electrochemical gradient. A regulated K^+ permeability with homoeostatic functions as delineated above and discussed previously (Kristensen, 1980; Schultz, 1981; Bakker-Grunwald, 1983) attracts interest as a mechanism of general importance in a variety of cell types.

The present paper deals with the mechanism of the increase in K^+ efflux from isolated rat hepatocytes during Na^+-coupled alanine uptake. The results indicate that the K^+ efflux occurs by a quinine-sensitive pathway and that an increase in the cell volume represents a major stimulus for the increased K^+ efflux.

Methods and materials

Hepatocytes were isolated by collagenase perfusion of livers from 48h-starved female Wistar rats (weight about 150g) as previously described (Kristensen *et al.*, 1983). The incubations were performed at pH7.4 and 37°C in a modified Krebs–Ringer bicarbonate buffer (280mosmol/kg). The buffer contained 2% (w/v) defatted bovine albumin, and had the following composition (mM): Na^+ 135, K^+ 4.8, Ca^{2+} 2.4, Mg^{2+} 1.2, Cl^- 117.2, HCO_3^- 25, $H_2PO_4^-$ 2.4, SO_4^{2-} 1.2. During the measurements 2.5mM-amino-oxyacetate was present to obtain complete inhibition of the first step in the intracellular metabolism of alanine, thereby rendering alanine non-metabolizable.

Centrifugation of the cells (from 300μl samples of the cell suspension) through silicone oil into 1M-$HClO_4$ was used to terminate the incubation at desired times during the experiments. With this procedure, the cellular water volume was determined as inulin-inaccessible ^3H_2O space in experiments with ^3H_2O and hydroxy[^{14}C]methylinulin as space markers. Cell counting was performed in a Neubauer counting chamber. Cellular alanine uptake was measured with [^{14}C]alanine. The efflux of $^{42}K^+$ was measured and the results were

analysed as previously described in detail (Kristensen, 1980).

Additional methodological details are given in previous papers (Kristensen, 1980; Kristensen *et al.*, 1983).

Materials

$^{42}K^+$ (chloride salt; sp. radioactivity about 50 Ci/mol at delivery) was from the Danish Atomic Energy Commission, Risø, Denmark. Other radioactive substances were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Collagenase (CLS II) was supplied from Worthington Biochemical Corp., Freehold, NJ, U.S.A., silicone oil AR 200 (density 1.04g/ml) from Wacker Chemie, München, Germany, and ouabain from Fluka A.G., Buchs, Switzerland. Apamin was a product from Serva Feinbiochemica, Heidelberg, Germany, and furosemide from Hoechst A.G., Frankfurt/Main, Germany. Quinine and amino-oxyacetate were from Sigma Chemical Co., St. Louis, MO, U.S.A.

Results and discussion

Fig. 1 illustrates that the uptake of alanine from an extracellular concentration of 10mM is accompanied within 20–30min by an increase in the cell volume to about 115% of the control value. At this time the intracellular alanine concentration had increased to a steady-state value of about 90mM, as also previously reported from this laboratory (Kristensen *et al.*, 1983). For a perfect osmometer (and extracellular osmolality of 290mosmol/kg), one would have expected an increase in the volume to about 130% of control when the transmembrane alanine concentration difference of about 80mM had been attained. Consequently, the result in Fig. 1 seems to indicate activation of mechanisms which counteract (albeit incompletely) the tendency to cell-volume increase during concentrative uptake of alanine in rat hepatocytes. Fig. 1 further illustrates that addition of 100mM-sucrose (with alanine present) immediately decreased the cell volume to a stable value slightly below the original control value.

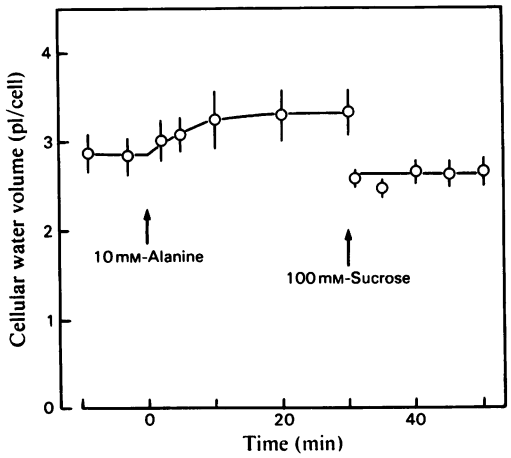

Fig. 1. *Changes in cellular water volume during alanine uptake*

Isolated rat hepatocytes were incubated in a buffer (280mosmol/kg) containing 135mM-Na^+ and 2.5mM-amino-oxyacetate. Alanine and sucrose (final concns. 10mM and 100mM respectively) were added as indicated by arrows. At appropriate times the cellular water volume (inulin-inaccessible ^3H_2O space) was measured as described in the Methods and materials section. Each point represents the mean of triplicate samples in three different cell preparations, with bars indicating ±S.E.M.

As studied in considerable detail in various other cell types, e.g. duck erythrocytes, lymphocytes and Ehrlich ascites-tumour cells, a major mechanism for regulatory volume decrease after cell swelling induced by extracellular hypo-osmolarity seems to be an increase in the cell-membrane permeability to K^+ and possibility to Cl^- (Kregenow, 1971; Grinstein *et al.*, 1982*b*; Hoffman *et al.*, 1984). Somewhat analogously, we have previously shown that the transport of alanine across the hepatocyte membrane is accompanied by a severalfold increase in the K^+ efflux (Kristensen, 1980). Bakker-Grunwald (1983) confirmed this observation and extended it by showing that osmotic cell shrinkage obtained by addition of 100mM-NaCl abolished the effect of alanine on the K^+ efflux. Since Na^+ and alanine are co-transported across the cell membrane, and the concentrative uptake of alanine is energized by the Na^+ electrochemical gradient (Kristensen, 1980), it is clearly desirable to evaluate such an effect of hyperosmolarity by employing a non-permeant non-electrolyte to change the osmolality. Therefore sucrose was chosen for this purpose, and it was established that 100mM-sucrose did not interfere with the concentrative uptake of alanine. As shown in Fig. 2, 10mM-alanine increased the rate constant of $^{42}K^+$ efflux from a control value of 0.015min^{-1} to 0.054min^{-1}. When the alanine-induced increase in cell volume was counteracted by addition of 100mM-sucrose, the rate constant of $^{42}K^+$ efflux was decreased from 0.054min^{-1} to 0.025min^{-1}, but did not completely return to the control value of 0.015min^{-1}. A modest effect of 100mM-sucrose on the rate constant of $^{42}K^+$ efflux was also observed in the absence of alanine. Thus the results in Figs. 1 and 2 indicate that the rate constant of $^{42}K^+$ efflux is dependent on the cell volume and that the effect of alanine on K^+ efflux is related to changes in the

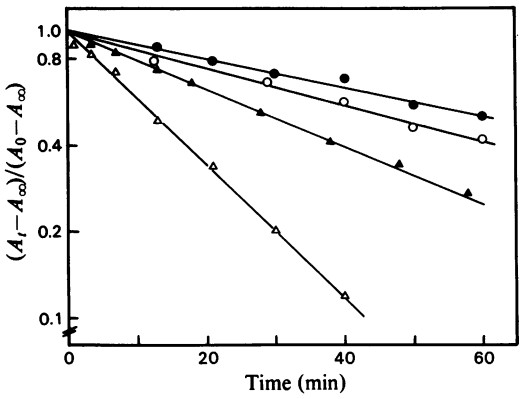

Fig. 2. *Effect of sucrose on the time course of $^{42}K^+$ efflux in the absence and presence of alanine*

After preloading of isolated rat hepatocytes with $^{42}K^+$ for about 60min, efflux was initiated at zero time by a 100-fold dilution of the extracellular $^{42}K^+$ radioactivity. Preloading and efflux were performed in the absence (○, ●) or presence (△, ▲) of 10mM-alanine in a modified Krebs–Ringer bicarbonate buffer, either under iso-osmolar conditions (○, △) or under hyperosmolar conditions (●, ▲) obtained by addition of 100mM-sucrose. At the times indicated, cellular $^{42}K^+$ radioactivity (A_t) was determined. Data are plotted according to the equation $A_t = (A_0 - A_\infty)\exp(-kt) + A_\infty$, which describes an exponential decay of radioactivity from its value at zero time (A_0) to a new lower equilibrium value at 'infinite' time (A_∞). Each point represents the mean of four experiments in different cell preparations.

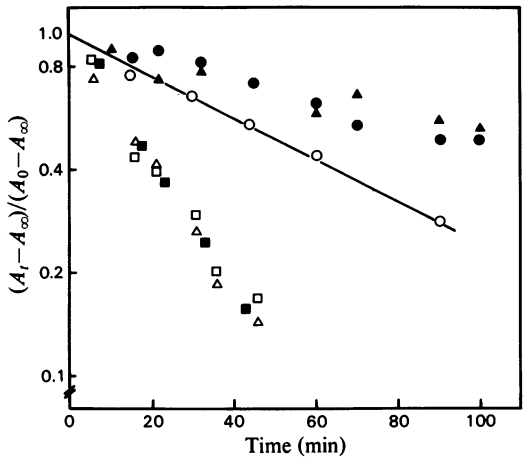

Fig. 3. *Effects of quinine, apamin and furosemide on the alanine-induced increase in the rate constant of $^{42}K^+$ efflux*

Isolated rat hepatocytes were preloaded with $^{42}K^+$, and the subsequent efflux was studied as described in the legend of Fig. 2. Two series were performed without addition of amino acids in the absence (○) and presence (●) of 1mM-quinine. In the remaining series 10mM-alanine was present, either alone (△) or together with the test substances, which were introduced 2min before initiation of the efflux and were then present throughout: ▲, 1mM-quinine; □, 0.3mM-furosemide; ■, 1μM-apamin. The points are from single representative experiments, except the control curve (○), which represents the mean of three experiments.

cell volume. That the alanine-induced K^+ efflux indeed contributes to cell volume regulation was strongly suggested by the finding that the cellular K^+ concentration (mmol/l of cell water) decreased from a control value of 165 ± 12 to 125 ± 13 (S.E.M., $n = 4$) after steady-state cellular accumulation of alanine from an extracellular concentration of 10mM. It should be noted that the effect of alanine on K^+ efflux is near-maximal at 10mM, but may be detected down to 0.5mM. Moreover, a similar effect is exerted in hepatocytes by several other amino acids (Kristensen, 1980), which together reach a concentration of 3–5 mM in the portal blood of rats (Ishikawa, 1977).

Broadly, our conclusions so far are in accordance with those reached by Bakker-Grunwald (1983). In contrast, Brown *et al.* (1983) concluded from their experiments with isolated rabbit enterocytes that the observed alanine-induced increase in K^+ efflux was not related to cell swelling, since the cellular volume (measured with 3-*O*-methylglucose) did not increase in the presence of 20mM-alanine. This interpretation might not be correct if the K^+ permeability is very sensitive to cell volume changes and the cell volume regulation is nearly complete. However, it is possible that factors other than the cell volume are the major signals which increase the K^+ permeability in enterocytes. Such unrecognized signals might also be a possible explanation for the difference in the present experiments (Fig. 2) between the $^{42}K^+$ efflux in control cells compared with cells accumulating alanine with sucrose present to avoid cell volume increase.

In lymphocytes and Ehrlich cells evidence has been presented that the K^+ efflux involved in regulatory volume decrease after hypo-osmotic cell swelling occurs via a Ca^{2+}-activated pathway (Grinstein *et al.*, 1982*a*; Hoffman *et al.*, 1984). In these cell types and in various other cells (Lew & Ferreira, 1978; Burgess *et al.*, 1981), Ca^{2+}-activated K^+ movements have been shown to be characterized by a marked inhibitory effect of quinine. Fig. 3 shows that the alanine-induced increase in $^{42}K^+$ efflux from rat hepatocytes could be completely blocked by 1mM-quinine. In contrast, no inhibitory effect was observed with the bee-venom neurotoxin apamin, which in some tissues, including guinea-pig liver, but not mam-

malian erythrocytes, acts as an inhibitor of Ca^{2+} activation of K^+ efflux (Burgess *et al.*, 1981). Furthermore, furosemide, which has been reported to inhibit a $1Na^+:1K^+:2Cl^-$ co-transport system in Ehrlich cells (Geck *et al.*, 1980), did not influence the alanine-induced $^{42}K^+$ efflux (Fig. 3). The possibility that quinine blocked the Na^+–alanine co-transport was ruled out by the finding that 1 mM-quinine inhibited alanine uptake by only 30% (results not shown).

In guinea-pig hepatocytes the presence of Ca^{2+}-activated channels has been strongly suggested (Burgess *et al.*, 1981) from experiments with the Ca^{2+} ionophore A23187 as a tool to increase cytosolic Ca^{2+}, and quinine as a tool to inhibit the activating effect of Ca^{2+}. However, parallel experiments with rat hepatocytes did not show an increase in K^+ efflux after addition of A23187, and higher concentrations of A23187 were associated with cell death. Using A23187, we have also tried to obtain a more direct support for the presence of Ca^{2+}-activated K^+ channels in rat hepatocytes, but the results were negative, like those reported by Burgess *et al.* (1981). The present results indicate as a new observation that a quinine-sensitive K^+-efflux pathway is activated during concentrative amino acid uptake in rat hepatocytes. However, considering the situation in rat hepatocytes discussed above, it remains a question whether the quinine-sensitivity reflects that the pathway is Ca^{2+}-activated.

It has been suggested by Spring & Ericson (1982) that the ion-transport processes involved in cell volume regulation after changes in osmolality might also serve other purposes, such as effecting responses to hormonal influences. The present study has shown that the concentrative cellular uptake of a metabolite may imply a marked tendency to cell swelling, triggering a sequence of events similar to that induced by hypo-osmolarity. One might therefore speculate whether a major physiological purpose of the cellular capacity for regulatory volume decrease is to avoid cell volume changes during uptake and metabolism of nutrients rather than to meet extreme changes in the extracellular osmolality.

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