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THE INFLUENCE OF AN UNCOUPLER ON AMINO ACID ACCUMULATION IN EHRLICH MOUSE ASCITES TUMOR CELLS

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Summary

In Ehrlich ascites tumor cells, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) acts at two different sites depending upon the concentration employed. (1) In non-glycolysing respiring cells, FCCP is seen to uncouple the mitochondria and thereby it inhibits the ATP-dependent (Na^+, K^+) pump. (2) In glycolysing cells, FCCP does not affect the electrogenic (Na^+, K^+) pump, but depolarizes the plasma membrane potential difference as visualized by the distribution of the lipid-soluble cation, tetraphenylphosphonium, and by an inhibition of the rheogenic, Na^+-dependent uptake of α-aminoisobutyric acid. A depolarization by FCCP also occurs under conditions where a K^+-diffusion potential is present and the pump is blocked by metabolic inhibition or by ouabain. Depolarization and FCCP-induced increase in H^+ fluxes across the plasma membrane exhibit a similar FCCP-concentration dependency. The imposition of proton-concentration differences in the presence of FCCP inhibits ($pH_i > pH_o$) or stimulates ($pH_i < pH_o$) α-aminoisobutyric acid uptake and tetraphenylphosphonium accumulation. The experiments indicate that FCCP shifts the plasma membrane potential of Ehrlich cells, which are normally relatively impermeable for protons, towards an H^+-diffusion potential.

Introduction

Ehrlich ascites tumor cells actively accumulate amino acids. The transport of neutral amino acids like the non-metabolizable α-aminoisobutyric acid

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Abbreviation: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

occurs as a rheogenic symport with Na^+, driven by the electrochemical potential difference of Na^+ [1,2]. The plasma membrane electrical potential difference (p.d.) is influenced by the electrogenic (Na^+, K^+) pump. In potassium-depleted sodium-rich cells, the high activity of the (Na^+, K^+) pump leads to a marked hyperpolarization stimulating α-aminoisobutyric acid uptake [3]. Gradual inhibition of the pump by increasing concentrations of ouabain depolarizes the plasma membrane potential difference, as visualized by the distribution of the lipid-soluble cation, tetraphenylphosphonium, and decreases α-aminoisobutyric acid accumulation [4]. Recently, it could be demonstrated that the uncoupler, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) inhibits α-aminoisobutyric acid accumulation in glycolysing cells without affecting the (Na^+, K^+) pump. The reduced accumulation of tetraphenylphosphonium under these conditions indicated a depolarization [5].

Another uncoupler, 2,4-dinitrophenol, decreases intracellular ATP levels due to uncoupling [6—8] and increases the proton translocation across the plasma membrane [9]. We therefore tried to separate FCCP effects on mitochondria and on plasma membranes and tested whether the FCCP-induced increase in proton permeability of the plasma membrane is responsible for the changes of the plasma membrane p.d. and subsequent variations in α-aminoisobutyric acid uptake. Furthermore, it was of interest whether FCCP is able to increase the proton permeability to such an extent that the electrical p.d. and α-aminoisobutyric acid uptake will be mainly determined by the proton-distribution ratio. If this is the case, variations of the proton gradient in the presence of FCCP will be an additional method for varying the driving forces of Na^+-dependent solute transport in Ehrlich cells. It should then also be possible to study the accumulation ratio of amino acids as a function of the chemical potential difference of sodium, when FCCP is used to short-circuit the electrical potential difference under conditions of an H^+ equilibrium. Such experiments could give further information on the energetics of the amino acid accumulation.

Methods and Materials

Cells. Ehrlich mouse ascites tumor cells were harvested from female Swiss mice by peritoneal aspiration 7—9 days after the inoculation of the tumor and suspended in Krebs-Ringer phosphate buffer, pH 7.4, containing 20.2 mM K^+, 134.4 mM Na^+, 2.55 mM Ca^{2+}, 1.36 mM Mg^{2+}, 139.7 mM Cl^-, 1.36 mM SO_4^{2-}, 10 mM phosphate, 1% bovine serum albumin and 12.5 μg/ml heparin. The cells were washed twice and then incubated for 30 min at 37° C in the same buffer but without heparin. This incubation yields 'potassium-rich cells', whereas the replacement of K^+ by Na^+ in the buffer and incubations for 15 min at 0° C and 15 min at 37° C decreases the potassium content to 10—20 mmol/l cell water and increases intracellular sodium levels to about 150 mM ('potassium-depleted cells').

α-Amino[^{14}C]isobutyric acid and [^{3}H]tetraphenylphosphonium uptake. If not stated otherwise, the reaction was started by the simultaneous mixing of cell suspensions with buffers containing the labelled α-aminoisobutyric acid, the final concentration being 100 μM. The uptake was terminated by cooling

the cell suspensions in ice water and centrifugation for 10 min at 2700 × *g*. [^{3}H]Tetraphenylphosphonium was added either simultaneously with the labelled amino acid or cells were incubated with tetraphenylphosphonium before the addition of the amino acid to ascertain an equilibration of the tetraphenylphosphonium distribution with the membrane p.d. at the end of the uptake experiment.

Intracellular electrolytes. Intracellular sodium and potassium were determined by flame photometry after extracting the freeze-dried cells with 3 ml distilled water. Chloride was measured after precipitating the protein in the extract with 3% trichloroacetic acid by colorimetric titration. Intracellular water content is calculated from the difference in weight of the packed cells before and after freeze-drying. Corrections for water trapped between the packed cells are made according to the method of Heinz and Mariani [10] assuming 0.16 ml of trapped water per g of wet packed cells.

Chemicals. α-Amino[^{14}C]isobutyric acid and [^{14}C]dimethyloxazolidine-2,4-dione were obtained from New England Nuclear. [^{3}H]Tetraphenylphosphonium was generously supplied by Dr. P. Geck. Valinomycin and bovine serum albumin were purchased from Serva, Heidelberg, FCCP from Boehringer, Mannheim.

Results

In the following experiments the effects of FCCP on the (Na^+, K^+) pump, α-aminoisobutyric acid uptake and tetraphenylphosphonium accumulation are demonstrated. Respiring cells are compared with antimycin A-treated, glycolysing cells to distinguish between the effects of FCCP on mitochondria and on the plasma membrane. Fig. 1, upper panel, shows the influence of increasing FCCP concentrations on the intracellular K^+ accumulation in respiring, non-glycolysing cells (curves a and c). K^+-depleted cells were incubated for 4 min with potassium to allow for intracellular potassium accumulation in the absence (curve a) and in the presence of ouabain (curve c). The difference between curves a and c represents the ouabain-sensitive K^+ uptake by the (Na^+, K^+) pump. This uptake is inhibited by increasing FCCP concentrations. The maximal inhibition is reached at about 5 μM FCCP.

The inhibition of the electrogenic (Na^+, K^+) pump by FCCP should lead to a decrease in the rheogenic, Na^+-dependent amino acid uptake. As shown in Fig. 1, lower panel, FCCP inhibits markedly the α-aminoisobutyric acid accumulation in the absence of ouabain (curve a), whereas it has no effect on α-aminoisobutyric acid uptake in the presence of ouabain (curve c). The difference between curves a and c represents the amino acid uptake driven by the electrogenic (Na^+, K^+) pump. FCCP at concentrations higher than 5 μM inhibits the pump-driven amino acid uptake completely.

Curves b and d in Fig. 1 are obtained with K^+-depleted cells treated with antimycin A, an inhibitor of the respiratory chain. The addition of 2.5 mM glucose to the incubation medium provided the cells with the substrate to maintain normal cellular ATP levels by glycolysis. FCCP does not affect the K^+ accumulation in the absence (b) or presence of ouabain (d). The difference between curves b and d remains constant over the whole FCCP concentration

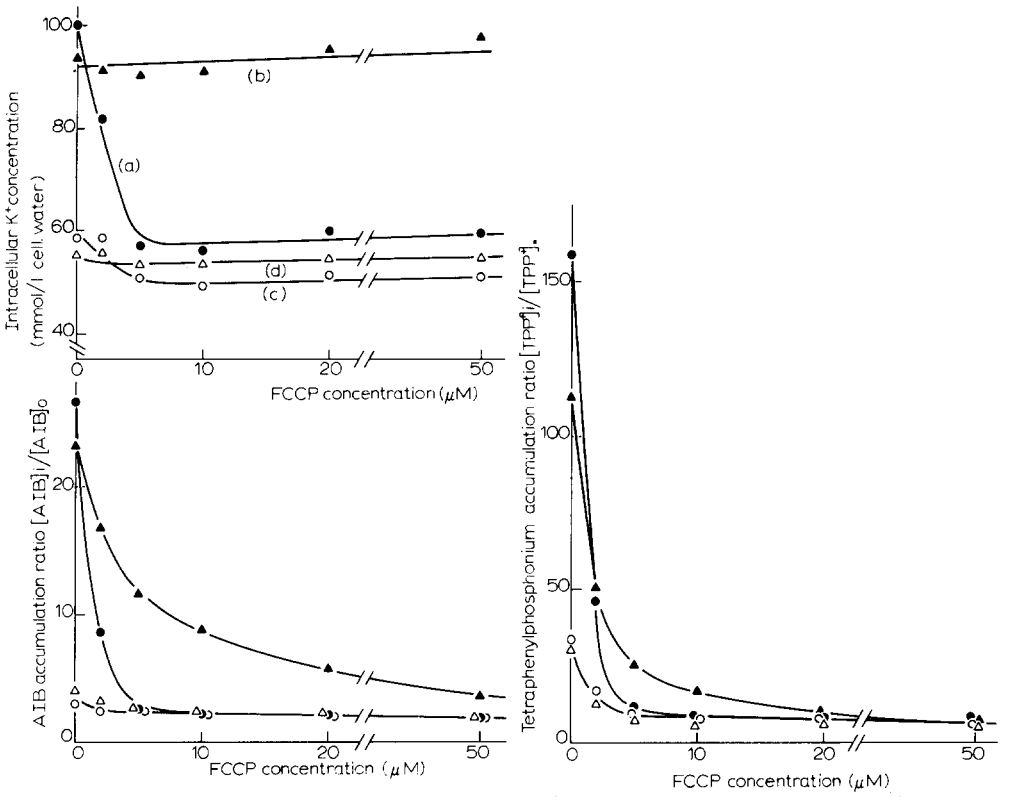

Fig. 1. The influence of FCCP on the ouabain-sensitive K^+ and amino acid accumulation in K^+-depleted, respiring cells and in non-respiring, glycolysing cells. Respiring cells: the difference between the values obtained without (curve a) and with 1.25 mM ouabain (curve c) represents the ouabain-sensitive uptake of K^+ and α-aminoisobutyric acid (AIB), respectively. To K^+-depleted EMAT cells suspended in K^+-free Krebs-Ringer phosphate buffer without glucose and pre-equilibrated with 10 μM [^{3}H]tetraphenylphosphonium, buffers were added containing K^+, 0.1 mM α-amino[^{14}C]isobutyric acid, [^{3}H]tetraphenylphosphonium and different amounts of FCCP. A second series contained 1.25 mM ouabain in addition. Final concentrations were 15 mM K^+, 0.1 mM α-aminoisobutyric acid, and 10 μM tetraphenylphosphonium. Electrolyte content, α-aminoisobutyric acid and tetraphenylphosphonium accumulation were determined after 4 min incubation at 37°C (for tetraphenylphosphonium results see Fig. 2). Glycolysing cells: the ouabain-sensitive accumulations of K^+ and α-aminoisobutyric acid are given by the difference between curves b (without ouabain) and d (with 1.25 mM ouabain). The cells were treated with 0.4 μM antimycin A to inhibit respiration and glycolysis was supported by addition of 2.5 mM glucose.

Fig. 2. The effect of FCCP concentration on the accumulation ratio of [^{3}H]tetraphenylphosphonium. The experimental details are described in Fig. 1. Curves a and c represent the results obtained with respiring cells in the absence of glucose, whereas curves b and d show the results with antimycin A-treated, glycolysing cells (cf. Fig. 1). TPP^+, tetraphenylphosphonium ion.

range. This indicates that in glycolysing cells the (Na^+, K^+) pump is not directly inhibited by FCCP. Likewise, the intracellular sodium and chloride concentrations are not affected by the uncoupler (not shown). The fraction of amino acid uptake, which is driven by the electrogenic component of the (Na^+, K^+) pump (difference between curves b and d in Fig. 1, lower panel), however, is reduced at higher FCCP concentrations. The maximal inhibition of α-aminoisobutyric acid uptake occurs at higher FCCP concentrations than in non-glycolysing, respiring cells. Amino acid uptake even at the highest FCCP con-

centration is not reduced to the same low level as in the assays containing ouabain. The activity of the (Na^+, K^+) pump creates a chemical Na^+ gradient more favorable for amino acid uptake in the assays without ouabain as compared to those with ouabain. This gradient increases α-aminoisobutyric acid uptake.

Fig. 2 compares the effects of FCCP on the tetraphenylphosphonium accumulation in respiring (a, c) and in antimycin A-treated, glycolysing cells (b, d). As in the preceding figures, the difference between the values in the absence (a, b) and in the presence of ouabain (c, d) represents the effect of the electrogenic (Na^+, K^+) pump. In the absence of FCCP, the (Na^+, K^+) pump increases the accumulation of tetraphenylphosphonium nearly 5-fold. The (Na^+, K^+) pump-dependent tetraphenylphosphonium accumulation in respiring, non-glycolysing cells is completely inhibited at 5 μM FCCP. In antimycin A-treated, glycolysing cells, however, 50 μM FCCP is needed to achieve the same inhibition of the pump-dependent tetraphenylphosphonium accumulation.

In further experiments, the influence of FCCP on plasma membrane p.d. and amino acid uptake was studied in cells which were energy depleted by blocking respiration with antimycin A and omission of glucose from the incubation medium. As under these conditions electrogenic pumps are not working, the plasma membrane p.d. will be determined only by ionic diffusion potentials. As K^+ is the most permeable ion, rheogenic amino acid uptake under these conditions should be influenced by variations of the K^+ concentration ratio. Fig. 3 shows the accumulation of α-aminoisobutyric acid and tetraphenylphosphonium in K^+-rich, energy-depleted cells, which were incubated with the labelled compounds for 4 min in buffers of various K^+ concentrations. The ratios of the K^+ concentrations were determined at the end of the uptake experiments and are probably smaller than the ratios at zero time as the gradients tend to decrease slowly with time. Fig. 3 shows that amino acid uptake and tetraphenylphosphonium accumulation increase with increasing K^+ concentration ratios. The addition of 40 μM FCCP causes a reduction of amino acid uptake at K^+ concentration ratios higher than about 2. FCCP also reduces the accumulation of tetraphenylphosphonium predominantly at higher K^+ concentration ratios. The uptake of α-aminoisobutyric acid into energy-depleted K^+-rich cells is also affected by FCCP when valinomycin is present (see also Fig. 4). This indicates that the uncoupler, even in the presence of valinomycin, is able to reduce the electrical potential difference across the plasma membrane. In line with this interpretation is the observation that FCCP strongly decreases the accumulation of a positively charged, permeant cyanine dye in the presence of valinomycin (Burckhardt, G., unpublished results).

Fig. 4 shows how the inhibition of amino acid and tetraphenylphosphonium accumulation depends on the concentration of FCCP. As valinomycin is present and cells are energy-depleted, amino acid uptake as well as the accumulation of tetraphenylphosphonium should depend on the magnitude of the K^+ concentration ratio. K^+-rich cells were incubated with the labelled compounds in the presence of valinomycin, antimycin A and without glucose. In separate assays, the distribution of [^{14}C]dimethyloxazolidine-2,4-dione was measured to calculate the apparent H^+ concentration ratio. As already seen in Fig. 3, FCCP decreases the accumulation of the amino acid and the lipophilic

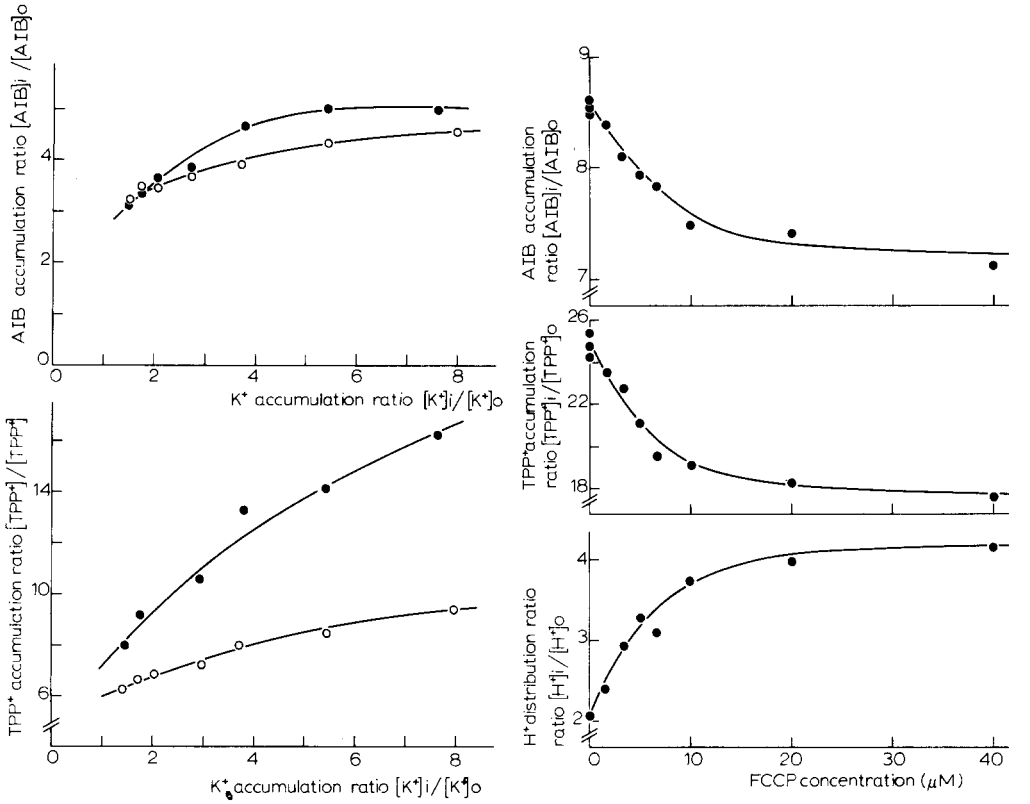

Fig. 3. The influence of different K^+ concentration ratios ($[K^+]_i/[K^+]_o$) on the accumulation of α-aminoisobutyric acid (AIB) and tetraphenylphosphonium (TPP^+) and the effect of FCCP. The K^+-rich cells were energy-depleted by 0.5 μM antimycin A and omission of glucose. (•) Controls without uncoupler; (○) in the presence of 40 μM FCCP. 2 ml of the cells were mixed with 8-ml buffers of various K^+ concentrations containing 0.1 mM α-amino[^{14}C]isobutyric acid, 10 μM [^{3}H]tetraphenylphosphonium and 1 mM ouabain. The sodium concentration in the medium was kept constant and KCl was substituted by tetraethylammonium chloride. Cells were allowed to accumulate the amino acid and tetraphenylphosphonium for 4 min at 37°C.

Fig. 4. FCCP concentration-dependent changes of the H^+-distribution ratio and of the accumulation of α-aminoisobutyric acid (AIB) and tetraphenylphosphonium (TPP^+) in energy-depleted cells. K^+-rich, energy-depleted cells (0.5 μM antimycin A, no glucose) were equilibrated with 10 μM [^{3}H]tetraphenylphosphonium for 4 min. The reaction was started by the addition of α-amino[^{14}C]isobutyric acid and—to parallel samples—[^{14}C]dimethyloxazolidine-2,4-dione in buffers with the indicated FCCP concentrations. The uptake was stopped 2 min later. All samples contained 20.2 μM valinomycin.

cation. The apparent H^+ ratio is increased from about 2 to more than 4. As in experiments with glycolysing cells (Fig. 1), rather high FCCP concentrations are needed to produce the maximal effects. The results also indicate that in the absence of FCCP the proton-distribution ratio must be lower than expected from the membrane potential. This could be explained by the low intrinsic H^+ permeability which prevents the protons equilibrating with the membrane potential within the incubation time.

FCCP was expected to increase the proton permeability in Ehrlich cells like other uncouplers. In order to test the concentration dependency of this effect, metabolically inhibited cells, which were incubated at pH 7.4, were

mixed with buffers of identical salt concentrations, but of lower pH, to yield an extracellular pH of 5.4, FCCP was present in various concentrations as indicated in Fig. 5. The reactions were stopped by cooling and centrifugation at different time intervals after the extracellular acidification. The proton uptake was calculated from the changes of extracellular pH, the pH values at zero FCCP concentration serving as controls. The FCCP concentrations needed for a maximal H^+ uptake are greater than 40 μM.

If high FCCP concentrations increase the proton permeability sufficiently with respect to other ion permeabilities, the plasma membrane p.d. should become a function of the H^+ concentration ratio. This was tested by sudden

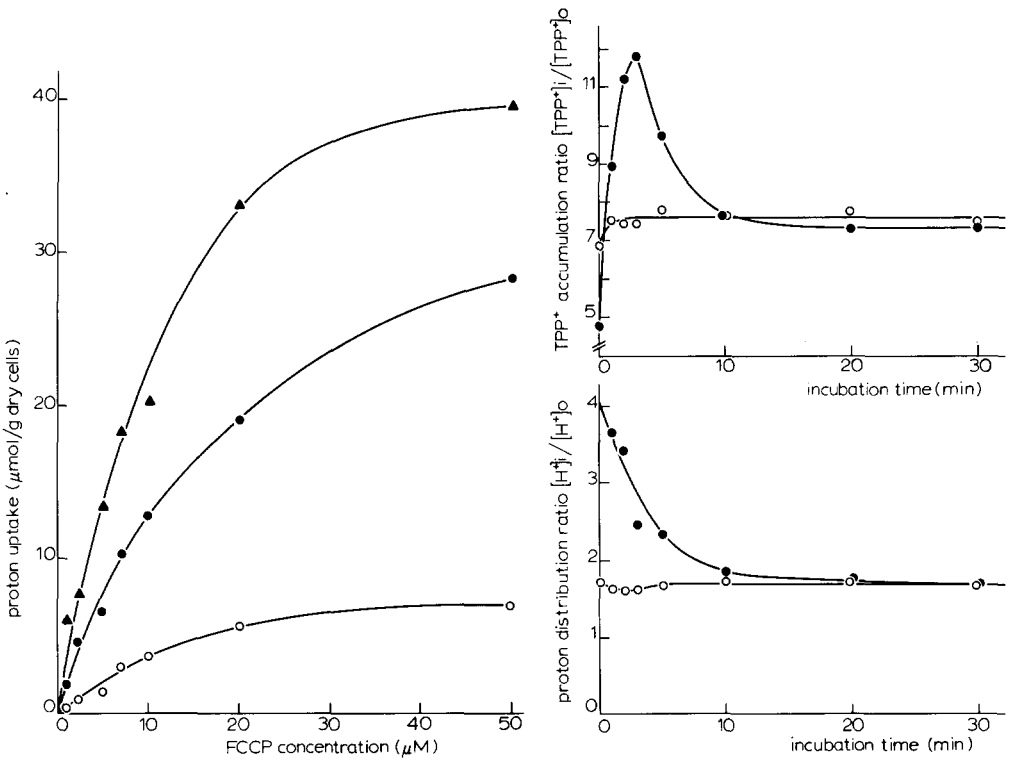

Fig. 5. Effects of various FCCP concentrations on proton uptake into energy-depleted cells after a sudden extracellular acidification. Cells were K^+-depleted and equilibrated at pH 7.4. At zero time, FCCP at the indicated concentrations, 0.5 μM antimycin A and [^{14}C]dimethyloxazolidine-2,4-dione were added simultaneously with 33 mM H_3PO_4 to give an extracellular pH change from 7.4 to 5.4. Proton uptake and [^{14}C]dimethyloxazolidine-2,4-dione distribution were stopped immediately (○), 2 (●), and 4 (▲) min after the extracellular acidification. After the cells were centrifuged, the supernatants were titrated with acid or base to find the buffer capacity of the incubation medium. The net proton uptake was calculated from the changes of the extracellular pH, the samples without FCCP serving as controls.

Fig. 6. Time course of changes of proton distribution ratio ($[H^+]_i/[H^+]_o$) and of tetraphenylphosphonium (TPP^+) accumulation after a sudden extracellular alkalinization in the presence of FCCP. K^+-depleted cells were divided into two portions, one of which is further incubated at pH 6.5, the other one serving as control at pH 7.4. 50 μM FCCP, [^{14}C]dimethyloxazolidine-2,4-dione and [^{3}H]tetraphenylphosphonium are present in both groups. The buffers contained no glucose in order to deplete intracellular ATP in the uncoupled cells. At zero time, the pH in both groups is brought to 7.4. At the given time intervals aliquots were removed to determine the distribution of dimethyloxazolidine-2,4-dione and tetraphenylphosphonium. (●) Cells preincubated at pH 6.5; (○) controls preincubated at pH 7.4.

changes of the extracellular pH. Energy and K^+-depleted cells were incubated in a K^+-free buffer, pH 6.5, for 30 min in the presence of 50 μM FCCP. Control cells were incubated at pH 7.4. At zero time the extracellular pH was brought to pH 7.4 by the addition of phosphate buffer. At the times indicated in Fig. 6, an aliquot of the suspensions was centrifuged and the distributions of tetraphenylphosphonium and dimethyloxazolidine-2,4-dione were determined. As can be seen from Fig. 6, the H^+ concentration ratio approached the same equilibrium as in the control group about 15 min after the extracellular pH change. This time course is comparable to that found by Garcia-Sancho and Sanchez [9]. The accumulation of tetraphenylphosphonium shows a sharp increase after the extracellular alkalinization with respect to the control group.

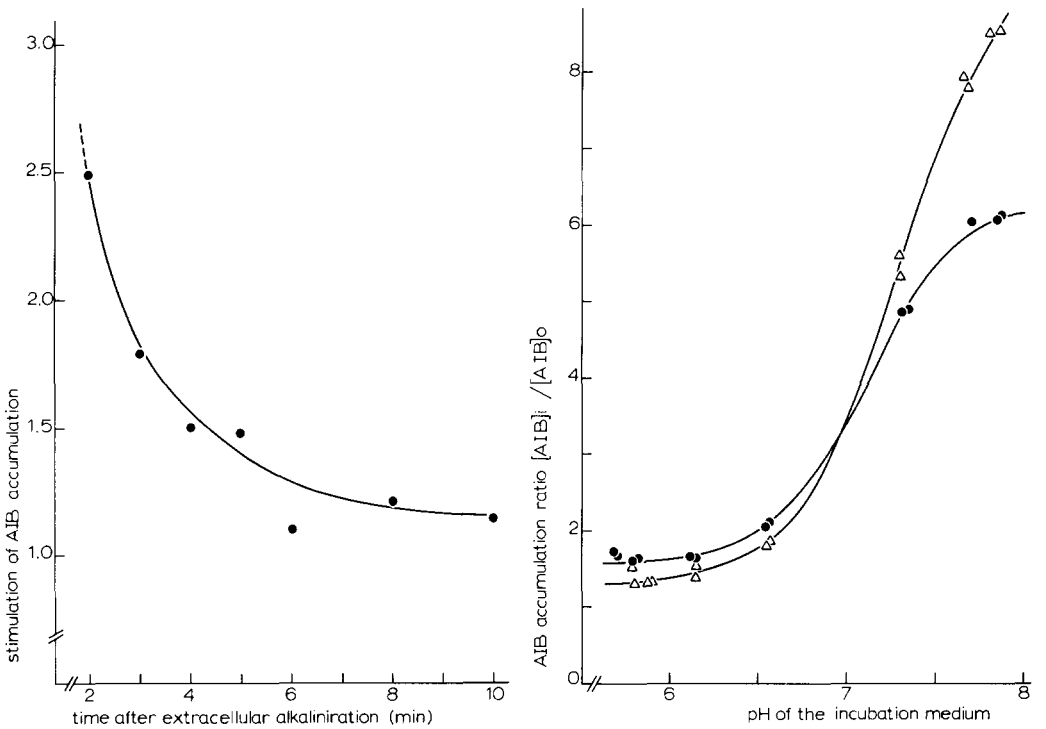

Fig. 7. Time course of the proton gradient stimulated α-aminoisobutyric acid (AIB) accumulation in energy-depleted cells after an extracellular alkalinization. Cells were either preincubated for 30 min at pH 6 or 7.4 in a K^+ buffer to yield K^+-rich cells. 50 μM FCCP was added before both groups of cell suspension were brought to pH 7.4. The buffers contained no glucose to deplete intracellular ATP in the uncoupled cells. Aliquots were taken at different times after the pH change and incubated with α-amino-[^{14}C]isobutyric acid for 2 min. The ordinate shows the accumulation ratio of α-aminoisobutyric acid in cells, which were preincubated at pH 6, divided by the α-aminoisobutyric acid ratios in cells preincubated at pH 7.4. The abscissa gives that time after the pH change at which the α-aminoisobutyric acid uptake was stopped.

Fig. 8. α-Aminoisobutyric acid (AIB) accumulation in K^+-rich cells as a function of a change of extracellular pH. 8 ml of K^+-rich cells, which were preincubated with a buffer at pH 6, were added to 2 ml buffer with α-amino[^{14}C]isobutyric acid to give the final pH values shown on the abscissa. 2 min later the reaction was stopped. Controls contain no FCCP (•), the other samples 50 μM FCCP (△). All samples contained 1 mM ouabain to avoid changes of the plasma membrane p.d. by the (Na^+, K^+) pump. Glucose was omitted.

As the distribution of tetraphenylphosphonium is slow, the accumulation lags behind the change of the H^+ concentration ratio.

An analogous experiment was carried out to see the influence of an extracellular alkalinization on amino acid uptake. If the plasma membrane p.d. in the presence of FCCP is influenced by the H^+ concentration ratio, an alkalinization of the extracellular medium with respect to the intracellular fluid should stimulate rheogenic amino acid uptake. The experiments were performed with K^+-rich cells, which contain a low intracellular sodium concentration and thus have a higher driving force for the Na^+-dependent amino acid uptake. All cells were energy-depleted (antimycin A, no glucose) to exclude influences of electrogenic pumps on the plasma membrane p.d. Cells were divided into two portions, one of which was incubated for 30 min at pH 6, the other at pH 7.4. After this incubation, the cells were further divided into several samples and the reaction was started by simultaneous addition of buffers to yield pH 7.4. Labelled α-aminoisobutyric acid was added at various times to the different cell suspensions and the uptake was stopped 2 min later. For any time after the buffer addition, the amino acid uptake in cells, which were brought from pH 6 to pH 7.4, was compared with that in the controls where no pH change took place. The data are given in Fig. 7. The imposition of an outwardly directed proton gradient stimulates the amino acid accumulation. The time course of stimulation resembles that of the H^+ ratio decay shown in Fig. 6.

The influence of FCCP on the accumulation of α-aminoisobutyric acid as a function of magnitude and direction of the imposed H^+ concentration difference is shown in Fig. 8. K^+-rich cells were incubated for 30 min at pH 6 and then divided into several aliquots. At zero time, buffers and labelled amino acid were added to give the final pH values indicated on the abscissa. Amino acid uptake was stopped 2 min later. The intracellular pH at the end of the preincubation at pH 6 was 6.4 as estimated from the distribution of dimethyloxazolidine-2,4-dione. In the absence of FCCP, amino acid accumulation is dependent on extracellular pH. It tends towards a maximum at pH 8. Below pH 6 there is only a small accumulation of the amino acid. Addition of 50 μM FCCP causes a small inhibition of amino acid uptake at extracellular pH values below 6.6, whereas at pH values above 7.2 a stimulation of amino acid accumulation can be seen. This indicates that the FCCP effect on α-aminoisobutyric acid is dependent on the magnitude and direction of the H^+ concentration gradient.

Discussion

As we have suggested previously [5], FCCP acts at two different sites: the mitochondria and the plasma membrane. Mitochondria are uncoupled at relatively low FCCP concentrations, as indicated by the inhibition of the ATP-dependent (Na^+, K^+) pump in the absence of glucose. The inhibition of the electrogenic (Na^+, K^+) pump leads to a depolarization of the plasma membrane and thereby reduces Na^+-dependent rheogenic amino acid uptake. These effects on the plasma membrane are secondary to the uncoupling of the mitochondria. The small K^+ uptake, which persists even at higher FCCP concentrations, does not cause a residual amino acid uptake. A possible explanation

is that FCCP treatment does not immediatly remove all ATP within the cell. The small hyperpolarization by the slowly working pump could easily be 'short-circuited' by the action of FCCP on the plasma membrane (see below). If the energy required for the pump is supported by glycolysis, the uncoupling of the mitochondria does not lead to an inhibition of the (Na^+, K^+) pump. In this case, the action of FCCP on the plasma membrane level can be demonstrated solely. It causes inhibition of α-aminoisobutyric acid accumulation, which requires much higher FCCP concentrations than uncoupling of the mitochondria. Thus, Fig. 1 shows that even with 50 μM FCCP a residual amino acid accumulation persists, which is driven by the sodium gradient established by the (Na^+, K^+) pump. As the intracellular sodium concentration in this experiment (Fig. 1) is independent of the uncoupler (not shown), the inhibition of amino acid uptake can either be due to a direct action of FCCP on the amino acid transport or to a reduction of the plasma membrane electrical potential difference. The experiments in Figs. 7 and 8, which show a stimulation of α-aminoisobutyric acid uptake by FCCP in the presence of a favorable H^+ gradient, rule out a direct inhibition of amino acid uptake by FCCP. Further evidence against a direct inhibition came from experiments with Li^+-loaded cells. Intracellular cations (Na^+ and K^+) in energy-depleted cells were nearly completely replaced by Li^+. Stepwise replacement of the extracellular Li^+ by Na^+ increased the Na^+ gradient directed into the cell and thereby Na^+-dependent α-aminoisobutyric acid uptake. It had no measurable influence, however, on the plasma membrane p.d. The permeability of the plasma membrane for Na^+ and for Li^+ is apparently of similar magnitude. Therefore, the changes in amino acid uptake observed should be directly related to changes in the chemical potential difference of Na^+. FCCP under these conditions showed no effect on α-aminoisobutyric acid uptake (Pietrzyk, C., unpublished result).

A depolarization of the plasma membrane electrical potential difference should decrease the accumulation ratios of tetraphenylphosphonium which is seen in Figs. 2—4. A quantitative interpretation of the data, however, is difficult, because the intracellular activity of this lipophilic cation is unknown. Moreover, tetraphenylphosphonium seems to accumulate in mitochondria because the accumulation ratios of tetraphenylphosphonium exceed the values expected from the K^+-distribution ratio (Ref. 1 and Geck, P., personal communication). FCCP would affect the accumulation of tetraphenylphosphonium at the mitochondrial level by uncoupling and at the plasma membrane level by depolarization. These two effects cannot be separated readily in Fig. 2. Valinomycin reduced the p.d. across the mitochondrial membrane and thereby the intramitochondrial accumulation of tetraphenylphosphonium. In this case, the observed decrease in tetraphenylphosphonium accumulation by FCCP in valinomycin-treated cells is indicative of a depolarization of the plasma membrane electrical potential difference (Fig. 4). Similar results are obtained with a cyanine dye which with FCCP present is accumulated less than in the absence of an uncoupler. Intramitochondrial dye accumulation [4,11,12] was prevented by the use of valinomycin (Burckhardt, G., unpublished results).

FCCP depolarizes the plasma membrane potential during the action of the (Na^+, K^+) pump or under conditions of a K^+ diffusion potential, most probably

by an increase in the proton permeability. Evidence for this hypothesis gave experiments which showed that inhibition of lipophilic cation and amino acid accumulation and increase in proton fluxes exhibit a similar FCCP concentration dependency. FCCP has to increase the proton permeability sufficiently with respect to other ions, especially K^+, in order to depolarize the plasma membrane potential difference even in the presence of valinomycin. As the accumulation of tetraphenylphosphonium and α-aminoisobutyric acid in the presence of FCCP depend on the magnitude and the direction of the proton gradient (Figs. 6—8) we conclude that the plasma membrane electrical potential difference under FCCP tends towards an H^+-diffusion potential. We are, however, not able to give a quantitative proof of this hypothesis because estimations of the plasma membrane potential by cyanine dye or tetraphenylphosphonium distribution are difficult. Also, the intracellular H^+ activity is unknown because intracellular compartmentalization influences the distribution of weak bases and acids like dimethyloxazolidine-2,4-dione [13].

The inhibition of amino acid uptake by FCCP in cells, where the (Na^+, K^+) pump is working, indicates that in these cells the H^+ distribution ratio H_i^+/H_o^+ is smaller than expected from the plasma membrane potential. This fits the observation of a lower intracellular H^+ concentration in metabolically active cells as compared to the extracellular H^+ concentration. For this reason an H^+ pump, which actively extrudes protons, was suggested [13]. Our observations are in agreement with recent findings of Thomas et al. [14], who showed that the plasma membrane is normally relatively impermeable to protons. Otherwise, protons would short-circuit the pump-generated electrical potential difference and thus reduce the driving force for Na^+-dependent amino acid uptake.

Similar findings to those we report for FCCP were recently reported by Johnstone [8] for 2,4-dinitrophenol, which at pH 7.4 at a concentration of $1 \cdot 10^{-4}$ M inhibited glycine uptake, but not when glucose was added. This effect is explicable by the uncoupling action of dinitrophenol on mitochondria. At pH 7.4, $1 \cdot 10^{-3}$ M dinitrophenol was needed to show effects similar to those reported here for high concentrations of FCCP on the plasma membrane (Pietrzyk, C., unpublished result). That at pH 6.5, $1 \cdot 10^{-4}$ M dinitrophenol sufficed to show inhibitory effects even when glucose was added indicates that at this lower pH dinitrophenol is more effective in depolarizing the plasma membrane potential and gives similar effects to high concentrations of FCCP. Besides the favorable proton gradient directed into the cell, the increased availability of the protonized species of dinitrophenol at lowered pH may have increased its effectiveness.

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