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Measurement of 'in situ' mitochondrial membrane potential in Ehrlich ascites tumor cells during aerobic glycolysis

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(1) A method is presented for continuous and simultaneous monitoring of the 'in situ' mitochondrial membrane potential ($\Delta\psi_m$) and respiration rate of Ehrlich ascites tumor cells. The method involves permeabilization of the plasma membrane, achieved by treatment with low digitonin concentration, and the use of a TPP^+ selective electrode attached to an oxygraph vessel. Binding of the probe inside the cells was analyzed assuming a proportional relationship between the amount of bound TPP^+ and the free concentration of the lipophilic cation. (2) Evidence is reported that the addition of glucose to digitonin-permeabilized Ehrlich ascites tumor cells causes a decrease of mitochondrial membrane potential that coincided with a transient enhancement of the respiration rate and remained unchanged during the subsequent Crabtree effect. We have characterized the effect of glucose on $\Delta\psi_m$ by determining its dependent on the glycolytic pathway and its sensitivity towards oligomycin. The mutual relationships between glucose and ADP effects on the mitochondrial membrane potential were also studied. A plausible mechanism underlying the depolarization of mitochondrial membrane induced by glucose is presented.

Introduction

The measurement of the membrane potential ($\Delta\psi_m$) in preparations of isolated mitochondria is of critical importance to verify the role of chemiosmotic proton circuits on several membrane-linked energy-dependent processes, such as oxidative phosphorylation or active transport across the mitochondrial membrane [1–3]. In the case of more complex systems, i.e., synaptosomes or intact mammalian cells, the quantification of the 'in situ' mitochondrial membrane potential can give valuable information on the functional relationships between the mitochondria and their surroundings in a particular metabolic state or under the influence of a given extracellular signal [4–7].

The quantitative estimation of $\Delta\psi_m$ in isolated mitochondria from the equilibrium distribution of an ionic probe, for instance lipophilic cations, is

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Abbreviations: DTNB, 5,5'-dithio-bis-(2-nitrobenzoic acid); TPP^+, tetraphenyl phosphonium; $TPMP^+$, triphenylmethylphosphonium; $\Delta\psi_m$, mitochondrial membrane potential.

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very well documented [8–13]. In contrast with isolated mitochondria, the uptake of lipophilic cations by several intact mammalian cells is a very slow process, and either long incubation periods or the addition of a lipophilic anion such as tetraphenylborate are required to achieve the equilibrium distribution of the mitochondrial membrane probe [14–17]. On the other hand, the distribution of these ionic probes between the cell and the incubation medium is determined by the mitochondrial membrane potential as well as by the plasma membrane potential. Hence, it is not possible to calculate $\Delta\psi_m$ without an adequate analysis of the compartmentation inside the cell. When this factor was taken into account, it became feasible to calculate the mitochondrial membrane potential of several mammalian cells from the equilibrium distribution of lipophilic cations [4,18,19]. Nevertheless, it should be noted that the amount of bound lipophilic cation was not accurately estimated in these papers and hence the reported values are probably overestimated [9].

Much effort has been devoted in the past to the biochemical characterization of tumor mitochondria in order to find some explanations for the high aerobic glycolysis usually present in tumors, as well as to ascertain the relative roles of glycolysis and respiration in the bioenergetics of cancer cells [20]. Since it is very well known that the addition of glucose to a tumor cell suspension oxidizing endogenous substrates causes significant changes of the respiration rate [21,22], it seemed interesting to study with more detail the mitochondrial membrane potential of tumor cells during aerobic glycolysis. At present few data are available on the magnitude of the 'in situ' mitochondrial membrane potential of tumor cells and its modification in different metabolic conditions. In the case of Ehrlich ascites tumor cells, safranine has been used as an optical probe of $\Delta\psi_m$, but only qualitative estimations of the mitochondrial membrane potential were reported [23].

In this paper we present a simple procedure for the continuous measurement of the 'in situ' mitochondrial membrane potential of Ehrlich ascites tumor cells that avoids the above-mentioned difficulties mostly caused by the plasma membrane. The method is characterized by (1) specific plasma membrane permeabilization by digitonin, (2) the use of a TPP^+-selective electrode that detects the uptake of the lipophilic cation by digitonin permeabilized cells, and (3) correction for TPP^+ binding assuming a model similar to that described for isolated rat liver mitochondria [9]. The method has been applied to measure $\Delta\psi_m$ during aerobic glycolysis in tumor cells. The results reported in this paper indicate that glucose specificaiiy induces a depolarization of the mitochondrial membrane. The effect of ADP and oligomycin on the mitochondrial membrane potential change brought about by glucose have also been examined.

Materials and Methods

Tumor cells. Ehrlich ascites tumor cells, hyperdiploid Lettré, were maintained by weekly transplantation in the peritoneal cavity of female Swiss albino mice aged two months. Ascitic tumors were centrifuged and the cells were washed twice with cold 154 mM NaCl, and suspended up to a density of $30 \cdot 10^6$ cells/ml in a medium of the following compositions: 6.16 mM KCl, 154 mM NaCl, 1.65 mM NaH_2PO_4, 9.35 mM Na_2HPO_4 (pH 7.4) [24].

Oxygen and TPP^+ uptake by tumor cells. A 2 ml thermostated vessel, equipped with a stirring device, a Clark-type oxygen electrode, a TPP^+-sensitive electrode [25] and a reference electrode, was used to measure the oxygen and TPP^+ uptake by intact or permeabilized cells. The signals from the oxygen and TPP^+-selective electrode were amplified and monitored in a dual-channel recorder. TPP^+ electrode was calibrated before the beginning of each experiment by successive additions of TPP^+ (0.25, 0.5 and 1.0 μM) to the Chance and Hess medium.

TPP^+ uptake by intact tumor cells was determined in long-term incubations carried out at 25°C in a metabolic shaker. Ascites tumor cells were suspended in the above-mentioned medium containing 1 μM TPP^+ up to a cell density of $15 \cdot 10^6$ cells/ml and incubated in 25 ml flasks under air atmosphere. At fixed times, 2 ml samples were withdrawn and transferred to the oxygraph vessel and the TPP^+ remaining in the medium was measured.

For the measurement of respiration and TPP^+ uptake by permeabilized cells, incubations were carried out in the same oxygraph vessel thermostatized at 25°C. $30 \cdot 10^6$ cells were incubated in Chance and Hess medium containing TPP^+ (1 μM) and digitonin (7.5–10 μM). Oxygen and TPP^+ uptake were measured continuously as before. TPP^+ uptake by permeabilized cells was steady until oxygen level in the incubation medium dropped to near zero. Catalase and limiting amounts of H_2O_2 were added when needed to maintain the oxygen level in the incubation medium.

Mitochondrial membrane potential measurements. Mitochondrial membrane potential of digitonin-permeabilized cells was calculated from the accumulation of the lipophilic cation after correcting for the binding of the probe essentially as described for isolated rat liver mitochondria. Due to the fact that in our experimental system the plasma membrane is permeabilized by digitonin treatment, a two-compartments model, intramitochondrial (I) and extramitochondrial (O), is assumed. The intramitochondrial compartment includes the matrix and the matrix side of the inner mitochondrial membrane, whereas the extramitochondrial compartment consists of the incubation medium, the cytosolic fraction and the rest of cellular membranes. The concentration of free TPP^+ in the intramitochondrial compartment, C_i, and in the extramitochondrial compartment, C_o, are related to the mitochondrial membrane potential according to the Nernst equation. C_o is the observable parameter in our experimental system and is continuously monitored with the TPP^+-selective electrode. C_i can be evaluated from the mass conservation equation for TPP^+ which states that the total amount of TPP^+ in the incubation vessel, N_T, is the sum of the free and bound forms of the lipophilic cation in both compartments. This equation can be expressed in several ways, depending on the procedure used to evaluate the binding of the potential probe to cellular constituents.

In this paper a proportional binding of the lipophilic cation has been assumed [9,13], the amount of bound TPP^+ in each compartment being proportional to the concentration of free TPP^+ in the same compartment. The proportionality constant, K, can be considered as an apparent partition coefficient of the lipophilic cation between the membrane and the aqueous phase in contact with it. If K_i and K_o are the partition coefficients for the intramitochondrial and extramitochondrial compartments, respectively, then the equation for TPP^+ conservation is:

$$N_T = C_o(V_o + K_o) + C_i(V_i + K_i) \quad (1)$$

where V_o and V_i are the volumes of the extra- and intramitochondrial compartments, respectively.

From Eqn. 1 it is possible to calculate C_i:

$$C_i = \frac{N_T - C_o(V_o + K_o)}{V_i + K_i} \quad (2)$$

and therefore,

$$\Delta\psi_m = \frac{RT}{F} \ln \frac{N_T - C_o(V_o + K_o)}{C_o(V_i + K_i)} \quad (3)$$

where R is the gas constant, T the temperature and F the Faraday constant.

We have assumed that the binding of the potential probe in the intra- and extramitochondrial compartments in our system can be considered similar to the binding to the internal and external side, respectively, of the inner mitochondrial membrane in Rottenberg's model. For rat liver mitochondria and TPP^+, $K_i = 7.9$ μl per mg protein, and $K_o = 14.3$ μl per mg protein [9]. In order to express these apparent partition coefficients in units of μl per $30 \cdot 10^6$ cells, the mitochondrial and cellular protein content of Ehrlich ascites tumor cells should be considered. According to available data [26], $30 \cdot 10^6$ cells contain 4.86 mg protein, of which 0.66 mg correspond to mitochondrial protein. From these values the partition coefficients are: $K_i = 5.21$ μl per $30 \cdot 10^6$ cells, and $K_o = 60.06$ μl per $30 \cdot 10^6$ cells. An intramitochondrial volume $V_i = 1.62$ μl per $30 \cdot 10^6$ cells was used in the calculation of the mitochondrial membrane potential [27].

Plasma membrane permeabilization by digitonin. Selective plasma membrane permeabilization of digitonin-treated cells was checked in parallel experiments by erythrosin staining of the cells and by measurement of the release of lactate dehydrogenase and citrate synthase, cytosolic and

mitochondrial marker enzymes respectively. $30 \cdot 10^6$ cells were aerobically incubated in 2 ml of Chance and Hess medium containing different digitonin concentrations (0, 5 and 10 μM) at 25°C. At fixed times, 0.1 ml samples of the incubation mixture were used to test the ability of the cells to exclude erythrosin. Different aliquots of 0.25 ml were centrifuged at 12000 r.p.m. for 2 min in an Eppendorf centrifuge, and the supernatants were assayed for the presence of marker enzymes. Enzymatic activities released in the supernatant were compared with those found after treatment of the cells with 1% Triton-X-100 in 0.33 M sucrose under similar incubation and centrifugation conditions. Lactate dehydrogenase [28] and citrate synthase [29] activities were measured in a Shimadzu Graphicord spectrophotometer at 25°C.

Aerobic glycolysis in digitonin-permeabilized Ehrlich ascites tumor cells. Ehrlich ascites tumor cells were aerobically incubated at 25°C in the Chance and Hess medium containing 5 mM glucose and various digitonin concentrations. 0.5 ml samples were withdrawn at different times and deproteinized with cold $HClO_4$. Neutralized perchloric extracts were used for the measurements of glucose and lactate by standard enzymatic procedures [30].

Chemicals. Glucose oxidase, dihydroxyacetone phosphate, 2-deoxyglucose, 2-deoxy-glucose-6-phosphate, malate, glutamate glutamine, ADP and digitonin were obtained from Sigma (St. Louis, USA). Peroxidase, lactate dehydrogenase, NAD^+, acetyl-coenzyme-A, oxaloacetic acid, 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) and the glycolytic intermediates were purchased from Boehringer (Mannheim, F.R.G.). TPP^+ was supplied by Fluka AG (Buchs, Switzerland). Erythrosin, glucose, lactate, succinate and citrate were obtained from Merck (Darmstad, F.R.G.).

Results

TPP^+ uptake by intact ascites tumor cells

The measurement of mitochondrial membrane potential by ion distribution techniques involves the estimation of the chemical probe concentration at both sides of the membrane after the thermodynamic equilibrium has been reached.

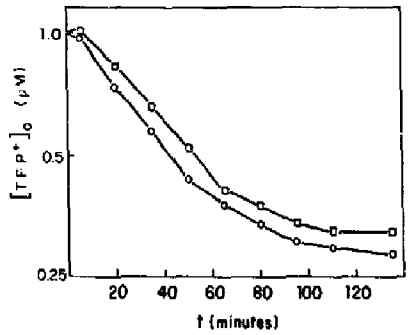

Fig. 1. TPP^+ accumulation by intact Ehrlich ascites tumor cells. $30 \cdot 10^6$ cells were aerobically incubated at 25°C in Chance and Hess medium containing 1 μM TPP^+, either in the absence (□) or in the presence (○) of 5 mM glucose. At the indicated times, aliquots of the incubation mixture were transferred to the oxygraph vessel and the TPP^+ remaining in the incubation medium was measured with the TPP^+ selective electrode.

Therefore, a rapid distribution of the lipophilic cation across the membrane is a prerequisite to monitor the membrane potential and its modifications in different experimental conditions. Experiments were done to test the ability of intact Ehrlich ascites tumor cells to take up TPP^+ from the incubation medium. As it is shown in Fig. 1, TPP^+ uptake by intact ascites tumor cells is a very slow process and it takes longer than 1 h before an apparent equilibrium distribution is observed. Glucose (5 mM) affected neither the kinetics nor the magnitude of TPP^+ uptake, the accumulation of the lipophilic cation in glycolysing cells being similar to that of cells respiring endogenous substrates. Different experiments showed that in cells incubated in the Chance and Hess medium for 2 h, the addition of glucose did not modify the equilibrium distribution of the lipophilic cation (results not shown). From these results it is not possible to exclude an effect of glucose on $\Delta\psi_m$, since the total TPP^+ accumulation in the cell depends on the magnitude of both mitochondrial and plasma membrane potential, besides the contribution of binding. On the other hand, any possible effect of glucose on the mitochondrial membrane potential would be difficult to observe due to the slow rate of equilibrium of the cationic probe between the medium and the cells.

TPP^+ uptake by Ehrlich ascites tumor cells in the presence of digitonin

To avoid these interferences of the plasma membrane on TPP^+ uptake and its distribution inside the cells, we decided to permeabilize the plasma membrane, without damaging the mitochondria, by using digitonin. In order to determine the digitonin concentration needed to permeabilize the plasma membrane, we measured the release of cytosolic and mitochondrial marker enzymes and the degree of erythrosin staining in cells incubated with different digitonin concentrations. As it can be seen in Fig. 2, the extent of cell permeabilization depends on both the digitonin concentration used and the length of the incubation period. Typically, after 15 min treatment with 10 μM digitonin, nearly 100% of the cells took up the erythrosin dye and the lactate dehydrogenase released after centrifugation at 12000 r.p.m. accounted for 80% of the total activity in the cells. In these conditions, citrate synthase in the 12000 r.p.m. supernatant was less than 5% of the activity released by treatment of the cells with Triton X-100. It should be pointed out that the high percentage of lactate dehydrogenase released in the incubation medium in the experiment depicted in Fig. 2 is due to the technique used for the separation of the cells from the medium. A minor percentage of the enzyme was detected in the medium when the cell suspension was filtered through Whatman glass microfibre filters (Results not shown). On the basis of these results we decided to use approx. 10 μM digitonin to permeabilize the plasma membrane. Nevertheless, the actual concentration used in the experiments reported in this paper slightly varied for different

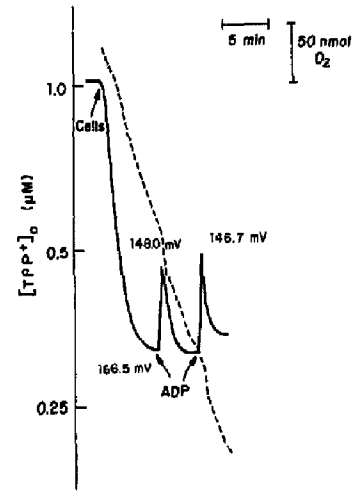

Fig. 3. TPP^+ and oxygen uptake by digitonin-permeabilized Ehrlich ascites tumor cells. $30 \cdot 10^6$ cells were incubated in Chance and Hess medium containing 2.5 mM succinate, 1 μM TPP^+ and 8 μM digitonin. ADP (40 μM) was added where indicated. TPP^+ uptake (solid line) and oxygen uptake (dotted line) were monitored by means of specific electrodes. Mitochondrial membrane potential values, expressed in mV, are indicated on the TPP^+ electrode record.

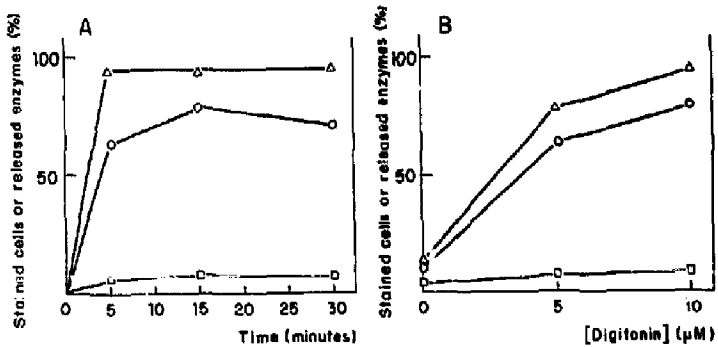

Fig. 2. Plasma membrane permeabilization by digitonin. (A) Time-course of cell permeabilization by 10 μM digitonin. (B) Effect of digitonin concentration on cell permeabilization after 15 min incubation. △, % erythrosin stained cells; ○, lactate dehydrogenase; □, citrate synthase. Enzyme activities released in the supernatant after centrifugation at 12000 r.p.m. are expressed as percentage of the total cellular activities.

cell batches, but always ranged between 7.5 and 10 μM.

Permeabilization of the plasma membrane by digitonin allows for uptake of charged substrates into the cell. Thus, it is possible to assess and to compare the mitochondrial membrane potential of cells respiring on different substrates and its modification by ADP. In Fig. 3 it is shown that TPP^+ accumulates very quickly in digitonin-permeabilized cells respiring succinate. Addition of 40 μM ADP induced a partial and reversible TPP^+ efflux and a transient increase of the respiratory rate. The same pattern was observed after a second addition of an equivalent amount of ADP. The effects of ADP on TPP^+ accumulation and oxygen uptake by digitonin-permeabilized cells are similar to those observed in isolated mitochondria during state 4–state 3 transition induced by ADP phosphorylation. These results also prove the selective permeabilization of the plasma membrane achieved by digitonin, since the changes caused by ADP are known to require the intactness of the mitochondrial inner membrane.

Change of the mitochondrial membrane potential induced by glucose

In digitonin-permeabilized cells respiring endogenous substrates, the uptake of TPP^+ from the medium is a rapid process as well (Fig. 4). Mitochondrial membrane potential, calculated from the equilibrium distribution of the lipophilic cation, was −161.7 mV. The addition of 5 mM glucose caused a rapid efflux of TPP^+ that coincided with a transient enhancement of the respiration rate. In comparison with the effect brought about by ADP, the efflux of TPP^+ induced by glucose was not reversible. On the contrary, a new distribution of the lipophilic cation was obtained simultaneous with the onset of the inhibition of oxygen uptake (Crabtree effect). Mitochondrial membrane potential in these conditions was −133.8 mV. The depolarization of the mitochondrial membrane induced by glucose was a highly repetitive phenomenon. Glucose induced a similar change of $\Delta\psi_m$, although smaller, in cells incubated with glutamine, a good respiratory substrate for tumor cells (Table I). It should be noted that aerobic glycolysis was not modified by digitonin

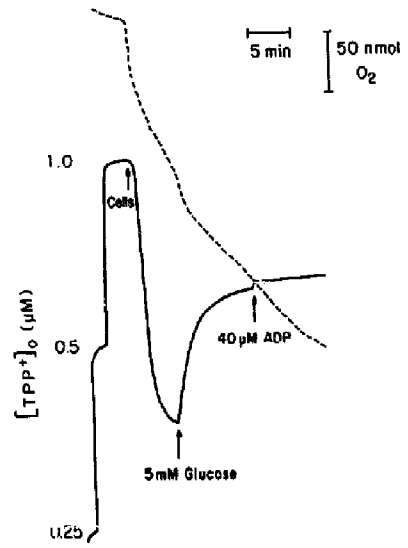

Fig. 4. Changes of TPP^+ and oxygen uptake induced by glucose in permeabilized tumor cells. Experimental conditions were similar to those described in the legend of Fig. 3 with the exception that no exogenous respiratory substrate was present in the incubation medium at the beginning of the experiment. Where indicated, 5 mM glucose and 40 μM ADP were added.

permeabilization of the cells, since no significant differences of glucose consumption and lactate production were found between control and per-

TABLE I

MITOCHONDRIAL MEMBRANE POTENTIAL OF PERMEABILIZED TUMOR CELLS INCUBATED IN DIFFERENT EXPERIMENTAL CONDITIONS

Ehrlich ascites tumor cells ($30 \cdot 10^6$ cells) were incubated in Chance and Hess medium containing TPP^+ (1 μM), digitonin (7.5–10 μM) and different substrates and effectors as indicated (5 mM). Glutamine was assayed at 2.5 mM. Mitochondrial membrane potential values are the mean of at least four independent experiments ± S.E.M.

Addition	$\Delta\psi_m$ (mV)
None	160.3 ± 5.0
Glucose	133.0 ± 1.7
Fructose	142.6 ± 2.4
2-Deoxyglucose	135.3 ± 7.3
Glucose + KF	140.6 ± 2.4
Glucose + alanine	132.6 ± 2.2
Glutamine	157.8 ± 3.8
Glutamine + glucose	148.3 ± 4.7

meabilized cells along 30 min incubation period (results not shown).

On the mechanism of mitochondrial membrane potential change caused by glucose

We have characterized the effect of glucose on $\Delta\psi_m$, mainly its relationship to the glycolytic pathway, from the measurement of the mitochondrial membrane potential in cells incubated with different sugars and glycolytic intermediates. Data shown in Table I indicate that other sugars such as fructose or 2-deoxyglucose, that induce respiration rate changes similar to those induced by glucose, also caused a significant depolarization of the mitochondrial membrane. On the other hand fluoride or alanine, inhibitors of enolase and pyruvate kinase respectively, did not reverse the effect of glucose.

To identify the enzymatic steps involved in the effect of glucose, permeabilized cells were incubated in the presence of different glycolytic intermediates and the mitochondrial membrane potential was calculated from the distribution of TPP^+. In addition, the effects of 2-deoxyglucose and 2-deoxyglucose-6-phosphate were compared. The results, shown as a crossover plot in Fig. 5, indicate that intermediate metabolites preceding phosphorylation steps in the glycolytic pathway, i.e., glucose-6-phosphate and fructose-6-phosphate, induced a change of mitochondrial membrane potential similar to that induced by glucose. Under the same experimental conditions, $\Delta\psi_m$ was not affected by the remaining glycolytic intermediates, with the exception of pyruvate that probably was used as a respiratory substrate. On the other hand, it is clear that 2-deoxyglucose caused a considerable depolarization of the mitochondrial membrane, whereas its phoshorylated derivative, 2-deoxyglucose-6-phosphate, did not modify the mitochondrial membrane potential.

The results in Fig. 5 strongly suggest that the phosphorylation of the sugar or glycolytic intermediate is a necessary step to observe the change of the mitochondrial membrane potential. Hence, it was assumed that the ADP produced in the phosphorylation reaction was translocated to the intramitochondrial compartment and used as a substrate of ATP synthase, ATP being used for

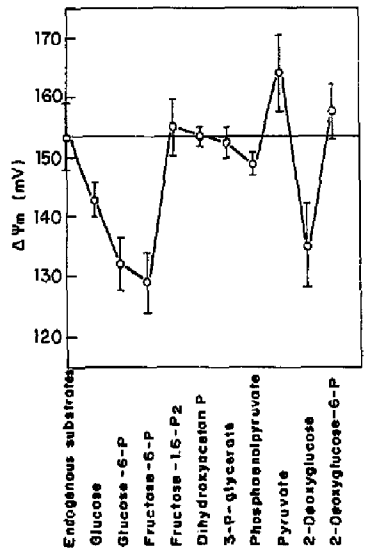

Fig. 5. Mitochondrial membrane potential of Ehrlich ascites tumor cells incubated with different sugars and glycolytic intermediates. Mitochondrial membrane potential was calculated from the TPP^+ accumulation in cells permeabilized by digitonin treatment (7.5–10 μM) incubated in the presence of different sugars. All the compounds were assayed at 1 mM in the incubation medium, except 2-deoxyglucose that was present at 5 mM. Values of mitochondrial membrane potential are the mean of at least three different experiments ± S.E.M..

the continuous phosphorylation of the sugar. To verify this hypothesis, we tested the sensitivity of glucose effect to oligomycin, an inhibitor of the

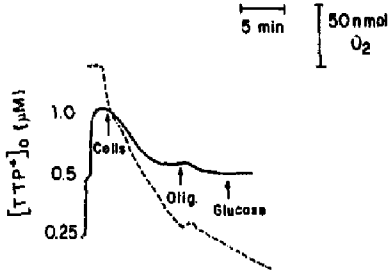

Fig. 6. Suppression of glucose effect by oligomycin. $30 \cdot 10^6$ cells were incubated in Chance and Hess medium containing 1 μM TPP^+ and 8 μM digitonin. TPP^+ uptake (solid line) and oxygen uptake (dotted line) by permeabilized cells were measured with selective electrodes. Where indicated, 3 μM oligomycin and 5 mM glucose were added.

mitochondrial ATP synthase. In Fig. 6 it is shown that the addition of oligomycin to cells oxidizing endogenous substrates slightly increased the accumulation of TPP^+ and caused an inhibition of oxygen uptake by the cells. In these conditions, glucose did not modify either the distribution of the lipophilic cation or the respiration rate.

Additional evidence favouring the proposed mechanism was obtained by testing the effect of ADP on mitochondrial membrane potential after the depolarization caused by glucose. The experiment depicted in Fig. 4 shows that the addition of 40 μM ADP to permeabilized cells incubated in the presence of 5 mM glucose did not change either the oxygen consumption or TPP^+ uptake. Similar results were obtained at higher ADP concentrations (up to 400 μM).

Discussion

Measurement of 'in situ' mitochondrial membrane potential

The estimation of mitochondrial membrane potential in mammalian cells by ion distribution methods is a much more complex task than in isolated organelles. Lipophilic cations have been used sometimes as indicators of the membrane potential in tumor cells, but without an adequate analysis of the compartmentation and/or binding of the probe inside the cells [14,18,31,32]. Although several experimental approaches have been used to solve the problems posed by the existence of multiple membranes in eukaryotic cells, most of them do not allow a continuous measurement of the distribution of the probe. It must be stressed that rapid and transient changes of mitochondrial membrane potential can only be measured if the equilibrium distribution of the ionic probe is attained and evaluated in a short time. Selective electrodes for lipophilic cations are suitable in the case of isolated mitochondria, where the equilibrium distribution of the probe is attained in a very short time. This is not the situation in the case of intact Ehrlich ascites tumor cells and TPP^+, as shown in Fig. 1. Until now these drawbacks have limited the application of ion distribution methods, particularly those using selective electrodes for lipophilic cations, to very few mammalian cells [7].

It is clear from the above considerations that in order to evaluate the mitochondrial membrane potential in tumor cells by ionic probes as TPP^+, an experimental approach should be sought that overcome those difficulties, mostly caused by the presence of the plasma membrane. Selective plasma membrane permeabilization by digitonin is an interesting method that has been previously used by different authors to test several mitochondrial processes 'in situ', i.e., calcium transport [26,33,34] or respiratory control [35,36]. This approach, in combination with the use of a TPP^+ selective electrode, allows the continuous measurement of TPP^+ uptake from the medium driven by the membrane potential of 'in situ' mitochondria without any interference of the plasma membrane. Moreover, digitonin treatment does not disturb the activity of the metabolic pathway, i.e., aerobic glycolysis, which effect on $\Delta\psi_m$ is under study.

The selective effect of digitonin relies upon the different cholesterol to phospholipid ratios in plasma and mitochondrial membranes. Experimental conditions must be carefully checked in the case of tumor cells because the cholesterol content of the mitochondrial membrane in these cells is higher than in normal cells [37]. At the digitonin concentrations used in this paper (between 7.5 and 10 μM) specific permeabilization of the plasma membrane is achieved while the mitochondrial functions are preserved. This has been inferred from different approaches: erythrosin staining of the cells, release of marker enzymes after centrifugation of the cell suspension, and also from the changes of oxygen consumption and TPP^+ uptake following the addition of ADP.

In digitonin-permeabilized cells, TPP^+ is expected to be distributed according to the mitochondrial membrane potential. In addition, the binding of TPP^+ to cellular constituents must be considered. In this paper we have assumed that the amount of bound TPP^+ in each compartment, intra- and extramitochondrial, is proportional to the concentration of the lipophilic cation in that compartment, the corresponding proportionality constants being K_i and K_o. This correction procedure has been applied to isolated rat liver mitochondria, and the $\Delta\psi_m$ values obtained with $TPMP^+$ and TPP^+ were in very good agreement

with the mitochondrial membrane potential calculated from ^{86}Rb distribution, particularly at membrane potentials higher than 120 mV (negative inside) [9].

The experimental system used in this study is more complex than isolated mitochondria. Hence, several simplifications and assumptions were made in order to calculate $\Delta\psi_m$ from the accumulation of TPP^+. Presumably, the binding of this lipophilic cation in the intramitochondrial compartment does not depend on the complexity of the extramitochondrial compartment and, therefore it can be reasonably described by the same partition coefficient K_i as used for isolated mitochondria. The evaluation of binding in the extramitochondrial compartment is more difficult to ascertain but if it can be also described by an apparent partition coefficient, the question arises of whether the K_o value used in this paper is the appropriate one for this purpose. However the accuracy in the value of K_o is not decisive for the calculation of $\Delta\psi_m$. In fact it can be shown that a 4-fold increase of K_o changes the calculated $\Delta\psi_m$ by about 3 mV. The reason for the small influence of K_o on $\Delta\psi_m$ values is that, according to Eqn. 1, the contribution of binding to the total accumulation of TPP^+ in each compartment is determined by the respective values of K and V. It is clear that in most experimental conditions $K_o < V_o$, but $K_i > V_i$. That means that the binding of the probe in the extramitochondrial compartment is minor compared to the amount of free TPP^+ in this compartment, whereas for the intramitochondrial compartment the binding is much more important. Hence, the calculated $\Delta\psi_m$ is less sensitive to a variation of K_o than to a change of K_i. The uncertainty of K_i sets therefore certain limits to the reliability of the absolute values of membrane potential. In a similar way it can be shown that the value of V_i has a small effect on the calculated $\Delta\psi_m$. As Rottenberg [9] pointed out this offers an advantage in experimental systems where the accurate measurements of intramitochondrial volume is not possible.

Change of $\Delta\psi_m$ induced by glucose

The results presented in this paper prove that glucose causes a decrease of the mitochondrial membrane potential in permeabilized Ehrlich ascites tumor cells respiring endogenous substrates. Indeed, the rapid efflux of TPP^+ from permeabilized cells observed in the presence of glucose could be due to different causes, i.e., release of endogenous substrates from the cells, with the consequent inhibition of respiration, or even to the permeabilization of the mitochondrial inner membrane caused by a possible excess of digitonin. The first alternative explanation was ruled out because digitonin did not modify the oxygen uptake of cells respiring endogenous substrates. In addition, the experiment in Fig. 4 indicate that TPP^+ efflux from permeabilized cells solely took place after the addition of glucose: no change of the distribution of the lipophilic cation along the incubation time was noticed in the absence of added glucose. Moreover, glucose also induced a similar decrease of TPP^+ accumulation in cells incubated with glutamine, which is a good respiratory substrate for these tumor cells (Table I). The fact that aerobic glycolysis was not modified by digitonin treatment also corraborates that digitonin-permeabilized cells can maintain high levels of cofactors and low-molecular-weight metabolites, confirming previous data obtained by Cockrell [26]. Finally, the kinetics of TPP^+ release brought about by glucose differed of the slower efflux of lipophilic cation that is observed after damaging the mitochondrial membrane with high digitonin concentrations. We therefore attributed the TPP^+ movement noted in the presence of glucose to a specific effect on the mitochondrial membrane potential.

The mechanism whereby glucose induces a depolarization of the mitochondrial membrane has been inferred from the measurement of 'in situ' $\Delta\psi_m$ in cells incubated in the presence of different sugars and glycolytic intermediates, as well as from the absence of any glucose effect in oligomycin-inhibted cells. The results reported here indicate that: (1) the flux through the complete glycolytic pathway is not necessary to induce the mitochondrial membrane potential change, but only a phosphorylation reaction of the sugar (as those catalyzed by hexokinase or phosphofructokinase) is a requisite. This fact explains the lack of sensitivity of the glucose effect to fluoride or alanine, since the respective target enzymes do not use ATP as substrate; and (2) the decrease of $\Delta\psi_m$

is caused by the mitochondrial phosphorylation of ADP previously produced in the cytosolic compartment, the newly formed ATP being used for further phosphorylation of the sugar.

Thus, according to our view, the change of mitochondrial membrane potential induced by glucose in permeabilized cells is similar to the depolarization caused by ADP phosphorylation in isolated mitochondria. In fact, a rapid increase of cellular ADP following glucose addition to intact Ehrlich ascites tumor cells was previously reported [30]. Moreover, an initial burst of oxygen uptake [22] paralleled the depolarization of mitochondrial membrane induced by glucose (Fig. 4) as would be expected if protonmotive force controlled the respiration rate in the form predicted by the chemiosmotic coupling mechanisms. Nevertheless, the stimulation of oxygen uptake was transient (1–2 min) because the Crabtree effect was quickly set up.

Part of the results presented in this paper are of some interest concerning the mechanism underlying the inhibition of respiration caused by glucose, which is a very common feature of many glycolysing tumor and normal cells. After the classical work by Chance and Hess [22], the view that the Crabtree effect is due to a deficiency of mitochondrial phosphate acceptor has been maintained by several authors [38,39]. If this hypothesis were correct, then the addition of ADP should reverse the inhibition of respiration rate caused by glucose in permeabilized cells. Our results do not support such possibility but, on the contrary, they suggest that there is an adequate supply of phosphate acceptor to the mitochondria of glycolysing cells. Recent experiments in our laboratory demonstrate that several respiratory substrates partially reverse the Crabtree effect and the change of mitochondrial membrane potential caused by glucose in digitonin-permeabilized tumor cells. These results would suggest that the availability of respiratory substrates, instead of ADP, is a limiting factor during the Crabtree effect.

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