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Monitoring of the Mitochondrial and Plasma Membrane Potentials in Human Fibroblasts by Tetraphenylphosphonium Ion Distribution[1]

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Abstract

The lipophilic cation tetraphenylphosphonium (TPP^+) is accumulated by human skin fibroblasts across both the plasma and mitochondrial membranes. We show here that TPP^+ uptake is indeed greatly decreased under conditions leading to de-energization of mitochondria. The TPP^+ accumulation in the presence of the proton ionophore FCCP has been used for determination of the plasma membrane potential across the plasma membrane, after correction for potential-independent binding of TPP^+ to cellular components. Following this procedure, a value of 75 mV has been obtained. Through the amount of TPP^+ released by FCCP treatment, an estimate of the *in situ* mitochondrial membrane potential has been made. Furthermore, we report that the mitochondrial component of TPP^+ accumulation decreases with aging of fibroblast cultures.

Key Words: Human fibroblasts; plasma membrane potential; mitochondrial membrane potential; tetraphenylphosphonium ion.

Introduction

Human skin fibroblasts represent a cellular system in which many biochemical processes have been thoroughly investigated. Owing to this and to the fact that fibroblasts can be easily obtained from biopsies and maintained in

[1] Abbreviations: $\Delta\psi_m$: membrane potential across the *in situ* mitochondria; $\Delta\psi_p$: membrane potential across the plasma membrane; TPP^+, tetraphenylphosphonium; HEPES: *N*-2-hydroxyethylpiperazine *N'*-2-ethanesulfonic acid; FCCP: carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

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culture, they have been widely utilized to investigate the molecular defects associated with several human genetic disorders (Seegmiller, 1976; Neufeld *et al.*, 1975; Brown and Goldstein, 1976; Rodemann and Bayreuther, 1986; Rugolo *et al.*, 1986b).

In this study we describe a method, based on distribution of the lipophilic cation tetraphenylphosphonium (TPP^+) between cells and medium, for determination of the membrane potentials across both the plasma and inner mitochondrial membranes in human fibroblasts. Indeed, fibroblasts are rather small cells, and the direct determination of the plasma membrane potential by means of microelectrodes, although technically feasible (Swift and Todaro, 1968; Villereal and Cook, 1978; Moolenaar *et al.*, 1982), is a rather difficult approach to be used as a routine method.

Lipophilic cations have previously been used for the estimation of changes in $\Delta\psi_p$ in neuroblastoma–glioma hybrid cells (Lichtshtein *et al.*, 1979), lymphocytes (Kiefer *et al.*, 1980), murine epidermal cell lines, and 3T3 fibroblasts (Seemann *et al.*, 1983). However, in all these reports, the $\Delta\psi_p$ was estimated by means of the TPP^+ distribution ratio obtained from total TPP^+ accumulation in low $[K^+]$ medium, after subtraction of the cation accumulation measured in a high $[K^+]$ medium. This quantitative approach contains two rather inappropriate assumptions, namely: (1) that $\Delta\psi_p$ is zero in high $[K^+]$ medium, and this is not true for $\Delta\psi_p$ with a significant Cl^- component (Felber and Brand, 1982), and (2) that the amount of TPP^+ taken up into mitochondria remains constant even when the plasma membrane is depolarized by high K^+ concentration. However, $\Delta\psi_p$ depolarization causes cytosolic $[TPP^+]$, and therefore mitochondrial $[TPP^+]$, to drop by an order of magnitude, even if $\Delta\psi_m$ remains unchanged (Felber and Brand, 1982). For this reason, assumptions (1) and (2) give an erroneous evaluation of $\Delta\psi_p$ and do not allow one to establish whether variations in TPP^+ accumulation are functional changes in either $\Delta\psi_p$ and/or $\Delta\psi_m$. In the present paper we show that indeed the lipophilic cation TPP^+ is accumulated in cultured human fibroblasts across both the plasma and mitochondrial membranes, since the TPP^+ accumulation ratio is greatly decreased under conditions leading to de-energization of mitochondria. We conclude that the TPP^+ mitochondrial uptake must be taken into consideration for any quantitative evaluation of the plasma membrane potential by means of lipophilic ion distribution. This conclusion appears also to be in accordance with previous considerations concerning the use of lipophilic ions for $\Delta\psi_m$ measurements in mitochondria both isolated and *in situ* (Nicholls, 1974; Hoek *et al.*, 1980; Scott and Nicholls, 1980; Davis *et al.*, 1981; Gallo *et al.*, 1984).

Materials and Methods

Cell Cultures

Human diploid fibroblasts, derived from biopsies of the upper arm, were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum (Gibco), 4 mM glutamine, and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin). Cells were grown in a humidified atmosphere of 5% CO_2 in 95% air at 37°C and used betwen the 6th and 30th passages. Cells were removed from stock flasks by trypsinization and prepared for the experiments by seeding approximately 4×10^4 cells into each 2 cm^2 well of a Nunc 24-well culture plate. Cells were then allowed to grow to confluence in 1 ml of growth medium for 7 days. The age of fibroblast cultures was expressed as passage number, i.e., the number of times the cells have been subcultured since the time of explantation.

Determination of [^{14}C]-TPP^+ Accumulation Ratio by Fibroblasts

Confluent monolayers were washed twice with 2 ml/well of the incubation medium (130 mM NaCl, 3 mM KCl, 1 mM Na_2HPO_4, 1.3 mM $CaCl_2$, 1.5 mM $MgSO_4$, 10 mM D-glucose, and 20 mM Na-HEPES, pH 7.4) and preincubated in 2 ml/well of the same medium in a 37°C water bath. After 10 min the medium was shaken from the wells and 0.25 ml/well of the incubation medium containing 1 μM [^{14}C]-TPP^+ (0.25 μCi/ml) was added in each well. Incubations were carried out at 37°C. At the end of the incubation time, aliquots of the incubation medium were taken for counting. The incubation was stopped by quickly decanting the medium and immediately washing twice the cells with 2 ml/well of ice cold 0.85% NaCl, according to the technique described by Gazzola *et al.* (1981). The wells were then drained and cells dissolved with 0.3 ml/well of 0.2 M NaOH (15 min at 55°C). Aliquots (0.2 ml) were collected for counting in 6 ml of Beckman MP scintillation cocktail containing 0.7% acetic acid. Protein content remaining in each well was determined according to the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Determination of total and extracellular spaces was performed using ^3H_2O (1 μCi/ml) and [^{14}C]-polyethylene glycol 4000 (1 μCi/ml) respectively (Zoccarato *et al.*, 1983). Determination of the [^{14}C]-polyethylene glycol volume allowed a correction for extracellular space to be made to TPP^+ apparent space. The TPP^+ accumulation ratio (TPP^+ in/out) was then calculated by dividing these corrected spaces by the intracellular volume, measured as the [^{14}C]-polyethylene glycol impermeable, ^3H_2O permeable space. The intracellular volume was found to be 4.2 μl/mg protein.

Determination of Extra- and Intracellular K^+ Concentration

Cells were incubated and treated exactly as described for TPP^+ accumulation except that aliquots of the lysate were dissolved in 1 ml of 1 mM EDTA, pH 7.4, 0.1% NaCl, and 0.9% Na-deoxycholate. The K^+ content was measured both in the cellular lysate and in aliquots of the incubation medium by atomic absorption spectroscopy.

Permeabilization of Fibroblasts with Digitonin

Cells were incubated in the absence or in the presence of [^{14}C]-TPP^+ for 30 min. Digitonin was added and left in contact with cells for 1 min. The medium was then collected and kept at 2°C. Enzymatic activities were determined in the medium and in fibroblasts carefully scraped from the well and solubilized in the assay medium containing 0.1% (v/v) Triton X-100. Lactate dehydrogenase and glutamate dehydrogenase assays were performed at 30°C in a Hitachi 100-40 spectrophotometer, as described by Scott *et al.* (1980).

Reagents

[^{14}C]-Tetraphenylphosphonium and [^{14}C]-polyethylene glycol 4000 were obtained from Radiochemical Center, Amerstam, U.K. Other reagents were from Sigma Chemicals.

Results

The Accumulation of TPP^+ by Human Fibroblasts

TPP^+, as other lipophilic ions, distributes according to a Nernst equilibrium across bilayer regions of membranes (Bakeeva *et al.*, 1970). In Fig. 1A the time course of the TPP^+ uptake into human fibroblasts is shown. It is apparent that the steady-state distribution ratio of the cation was achieved within 30–40 min and a value of 75 was obtained. When the TPP^+ distribution ratio was plotted as a function of the external TPP^+ concentration, an optimal accumulation of the cation was obtained at TPP^+ concentrations below 2 μM. At higher concentrations, a significant reduction of TPP^+ accumulation ratio occurred (Fig. 1B), probably due to mitochondrial depolarization (Nicholls *et al.*, 1982).

It is widely established that the weak acid carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) completely abolishes the mitochondrial membrane potential (Nicholls, 1974). Furthermore FCCP has been assumed not to affect the plasma membrane potential in several cellular systems,

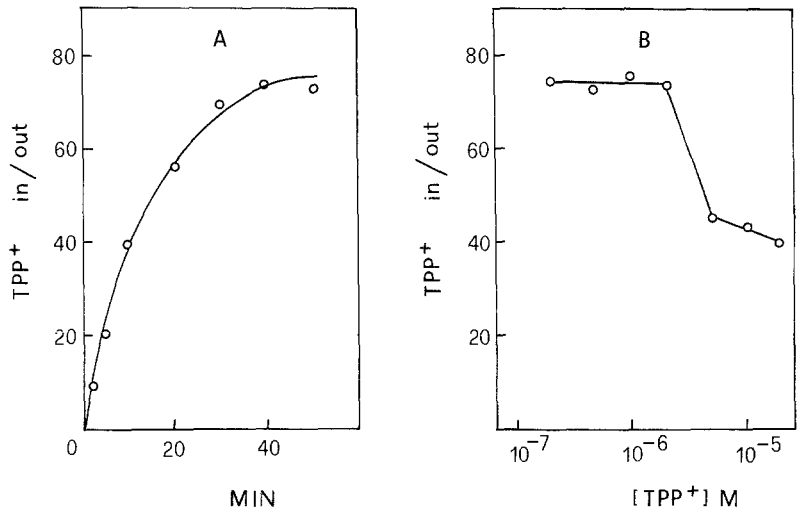

Fig. 1. TPP^+ accumulation within human fibroblasts as a function of time (A) and TPP^+ concentrations (B). The TPP^+ accumulation ratio was measured as described under Materials and Methods. In Fig. 1B, the $[^{14}C]$-TPP^+ accumulation ratio was determined at 40 min; each experimental point is the mean value of four determinations.

although a direct demonstration, by means of electrophysiological techniques, has not been provided so far. However, this assumption arises from experiments in which the steady-state distribution of $^{86}Rb^+$, which estimates the plasma membrane potential, was found not to be changed after addition of the uncoupler (Scott and Nicholls, 1980; Felber and Brand, 1982; Gallo *et al.*, 1984). Figure 2A shows that the steady-state accumulation ratio of TPP^+ in the presence of 4 μM FCCP was 27. The same result was obtained when the proton translocator was added to fibroblasts after the TPP^+ steady-state accumulation was achieved, allowing us to conclude that the distribution of TPP^+ between cells and medium is reversible and responds to manipulation of $\Delta\psi_m$.

The pattern of the TPP^+ accumulation as a function of FCCP concentrations is shown in Fig. 2B. From this it can be concluded that FCCP concentrations greater than 2 μM were required to completely abolish TPP^+ accumulation within mitochondria.

The glycoside rotenone is a well-known inhibitor of the mitochondrial NADH-coenzyme Q reductase (Singer and Gutman, 1971). As shown in Fig. 3A, addition of 4 μM rotenone only slightly affected TPP^+ accumulation by human fibroblasts. Note that in these experiments the TPP^+ accumulation ratio was > 100 (see below). This result is quite interesting because it indicates that, similarly to Ehrlich ascite cells and synaptosomes (Åkerman, 1979;

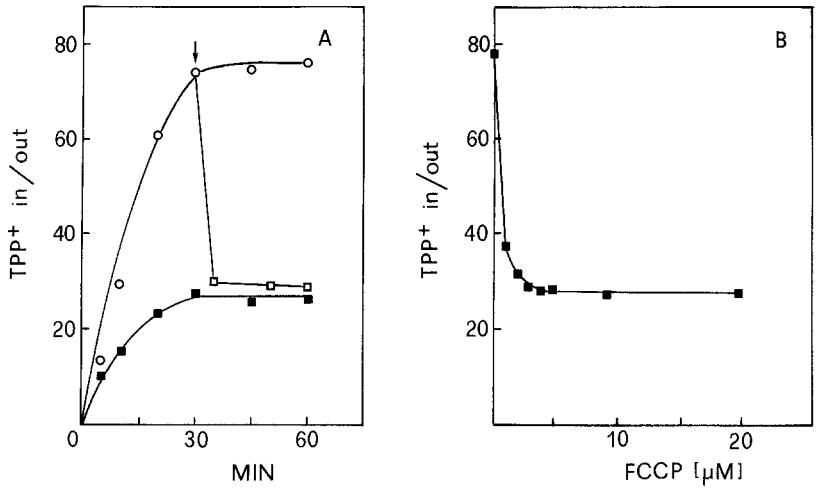

Fig. 2. Effect of FCCP on TPP^+ accumulation ratio. (A) Symbols: control (○); 4 μM FCCP added at $t = 30$ min (□); 4 μM FCCP added at $t = 0$ (■). (B) The TPP^+ accumulation ratio was determined at 40 min. Each experimental point is the mean value of at least four determinations.

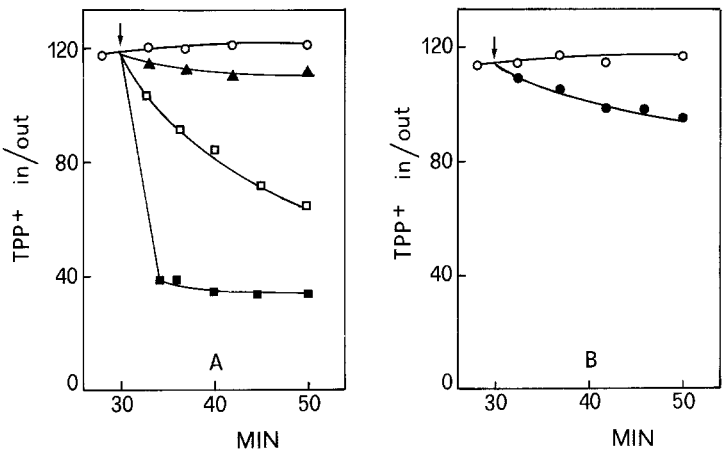

Fig. 3. Effect of rotenone, oligomycin, and ouabain on the steady-state TPP^+ accumulation ratio into human fibroblasts. (A) Additions: (○) none; (▲) 4 μM rotenone; (□) 2 μg/ml oligomycin; (■) 4 μM rotenone plus 2 μg/ml oligomycin. (B) (○) none; (●) 0.2 mM ouabain. The effect of the inhibitors was tested in five different experiments.

Scott and Nicholls, 1980), anaerobic glycolysis by fibroblasts also seems to be able to supply a cytosolic ATP-pool level sufficiently high to reverse the ATP-synthetase and maintain the energized state of the inner mitochondrial membrane. If this is the case, the simultaneous addition of rotenone and oligomycin would reduce the TPP^+ accumulation ratio to values close to

those obtained in the presence of FCCP as a result of complete abolition of $\Delta\psi_m$, and this is shown in Fig. 3A.

The addition of oligomycin alone would be expected to only affect the ATP-synthesis and hydrolysis, while $\Delta\psi_m$ is maintained by respiratory electron transport (Scott and Nicholls, 1980; Nicholls *et al.*, 1982). However, as shown in Fig. 3A, this expectation was not confirmed, and a partial decrease in TPP^+ accumulation ratio was observed upon oligomycin addition. A similar effect has been reported to occur also in pig lymphocytes (Brand and Felber, 1982), where it has been attributed to inhibition of the (Na^+,K^+)-ATPase by oligomycin (see also Fahn *et al.*, 1966). Figure 3B shows that the (Na^+,K^+)ATPase inhibitor, ouabain, reduces the TPP^+ accumulation ratio, although to a lower extent than oligomycin. In the presence of oligomycin, however, we failed to observe a further reduction in the TPP^+ accumulation ratio upon addition of ouabain (not shown), and this might be considered an indirect evidence of the (Na^+,K^+)ATPase involvement.

In conclusion, the effect induced by ouabain indicates that in human fibroblasts the $\Delta\psi_p$ is affected by the electrogenicity of the (Na^+,K^+)ATPase activity.

In Fig. 4, the TPP^+ accumulation ratios are plotted as a function of *in vitro* ageing of fibroblast cultures, expressed as the number of passages of fibroblast cultures. It can be observed that in cultures at early passage number (9 to 15), the TPP^+ accumulation ratios ranged between 101 and 129. By increasing the passage number, the TPP^+ accumulation ratio decreased and a value of 40 was determined in cultures at late passage number (> 25). It must be noted that the total protein content per well was not significantly influenced by increased passage number. Figure 4 also shows that the TPP^+ accumulation ratios measured in the presence of FCCP were slightly influenced by increased passage number.

Determination of Mitochondrial Membrane Potential

The above reported data tend to demonstrate that the magnitude of mitochondrial membrane potential can be indirectly determined from the total TPP^+ accumulation ratio after subtraction of TPP^+ accumulation in the presence of FCCP. This latter assumption is based on the fact that FCCP does not significantly affect the plasma membrane potential, as previously shown by Scott and Nicholls (1980), Felber and Brand (1982), and Gallo *et al.* (1984), although some effects on cytoplasmic pH have been reported (Arslan *et al.*, 1985). In order to know the concentration of TPP^+ in mitochondria, determination of mitochondrial volume is required. Of the total fibroblasts protein content, about $12 \pm 3\%$ can be recovered as isolated mitochondria following conventional centrifugation; the matrix volume of

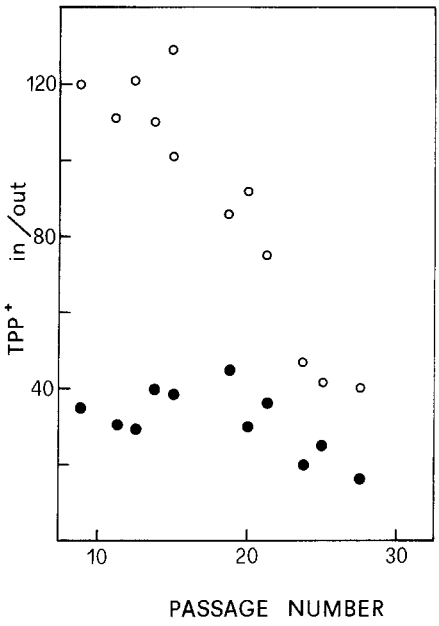

Fig. 4. Effect of *in vitro* aging on the TPP^+ accumulation ratio. The [^{14}C]-TPP^+ accumulation ratio was measured at 40 min. *In vitro* aging is expressed as cell passage number. Additions: (○) none; (●) 4 μM FCCP. Each experimental point is the average value of at least four determinations.

isolated mitochondria (measured as [^{14}C]-polyethylene glycol impermeable, ^3H_2O permeable space) was 0.8 μl/mg protein. From these data, the TPP^+ accumulation ratio within *in situ* mitochondria was estimated, and $\Delta\psi_m$ of 175 $\pm$ 5 mV was obtained in fibroblasts (at culture passage number $< 15 \pm 1$); this membrane potential is in good agreement with the values reported in other cell systems (Hoek *et al.*, 1980; Scott and Nicholls, 1980).

Potential-Independent Binding of TPP^+

Two experimental approaches have been used to determine the potential-independent binding of TPP^+ to cellular components, namely: (i) By assuming that $\Delta\psi_p$ is equal to the K^+ diffusion potential (Falber and Brand, 1982), the TPP^+ accumulation ratios were determined at defined extracellular K^+ concentrations. In Fig. 5, the relationship obtained between TPP^+ and K^+ accumulation ratios is shown; the intercept equivalent to 7- to 8-fold accumulation ratio corresponds to the potential-independent binding of TPP^+ to the cells; (ii) The steady-state value for TPP^+ accumulation by fibroblasts has been determined following permeabilization of the cell plasma membrane, as

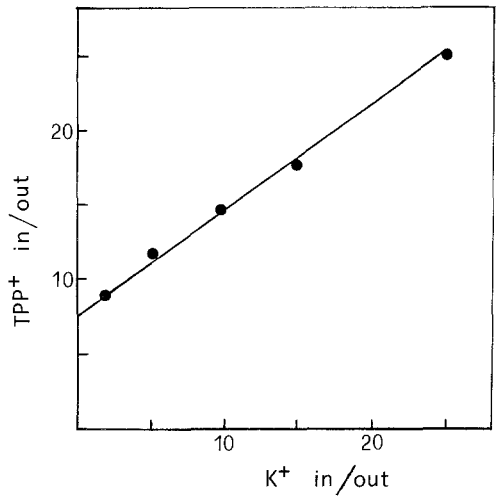

Fig. 5. Relationship between TPP^+ accumulation ratio and K^+ diffusion potential. The [^{14}C]-TPP^+ accumulation ratio was determined at 40 min in incubation media containing various concentrations of NaCl and KCl. For each experimental point the concentration of [Na^+] plus [K^+] was 133 mM in the presence of 25 μM valinomycin. Intracellular [K^+] was 160 mM.

previously shown by Gallo *et al.* (1984). In this study we have used the detergent digitonin, which disrupts cholesterol-containing membranes (Fiskum, 1985). Plasma membrane permeabilization was followed by measuring the release of the cytosolic enzyme lactate dehydrogenase in the incubation medium (Rugolo *et al.*, 1986a). As shown in Fig. 6A, upper trace, the maximum release of the enzyme was observed at about 80–100 μg/ml. Under these experimental conditions, glutamate dehydrogenase activity, a mitochondrial marker enzyme, was negligible (less than 7% of total activity) and not significantly affected at any digitonin concentrations (Fig. 6A, lower trace), indicating that mitochondrial membranes are still intact. The protein content per well remained unchanged throughout a digitonin concentration range of 5–100 μg/ml. Figure 6B, upper trace, shows that at digitonin concentrations up to 20 μg/ml, no appreciable loss of TPP^+ from fibroblasts was observed. At higher concentrations of digitonin, there was a progressive loss of TPP^+ until a minimum value was reached at 80–100 μg/ml digitonin. As shown in Fig. 6A and B, the digitonin-dependent release of lactate dehydrogenase from fibroblasts shows a good correlation with that of TPP^+. The effect of digitonin on the amount of TPP^+ associated with cells incubated in the presence of the uncoupler FCCP is shown in Fig. 6B, lower trace. It can be noticed that at digitonin concentrations higher than 50 μg/ml, the TPP^+ accumulation ratio reached a minimum constant value (potential-independent binding), which is

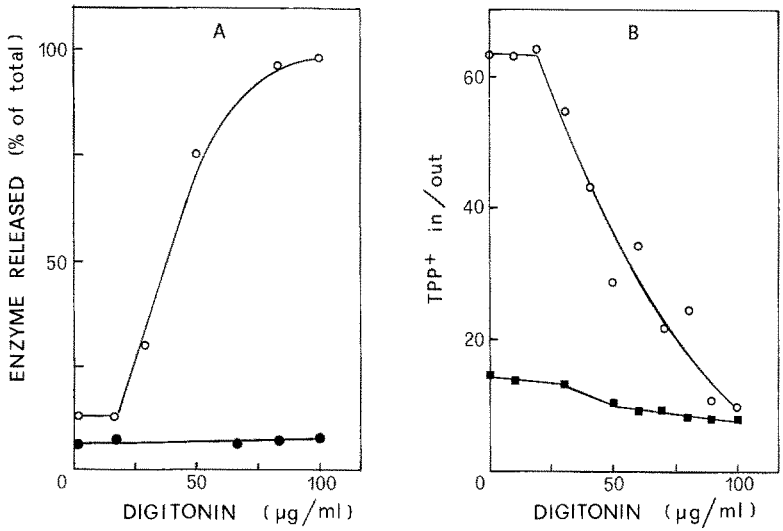

Fig. 6. The effect of digitonin concentrations on the release of some cellular enzymes (A) and on the TPP^+ associated with human fibroblasts (B). (A) Fibroblasts were incubated in the presence of varying concentrations of digitonin. Enzymatic activity, assayed in the medium, was expressed as percent of total activity determined in fibroblasts solubilized in 0.1% Triton X-100. (○) Lactate dehydrogenase activity; (●) glutamate dehydrogenase activity. (B) [^{14}C]-TPP^+ was determined at 40 min in the absence (○) and in the presence of 4 μM FCCP (■).

very similar to that observed in the absence of FCCP at 80–100 μg/ml digitonin. These results therefore indicate that maximal depolarization occurs in fibroblasts treated with 80–100 μg/ml digitonin alone. Under these experimental conditions the TPP^+ distribution ratio is close to 8 and the complete depolarization of mitochondria is likely to be due to the action of digitonin, which induces a sudden exposure of mitochondria to the millimolar free calcium concentration of the incubation medium (Scott *et al.*, 1980).

Determination of the Plasma Membrane Potential and Its Effect on TPP^+ Accumulation Ratio

The TPP^+ accumulation ratio obtained in the presence of the uncoupler FCCP can be substituted into the Nernst equation to quantify the plasma membrane potential, after correction for the potential-independent binding component. Following this procedure, a value close to 75 mV was obtained. The relative contribution of K^+ and Cl^- to fibroblasts $\Delta\psi_p$ was measured by determining the TPP^+ accumulation ratio in synthetic media of various ion concentrations by iso-osmotic substitution. Figure 7A shows the TPP^+

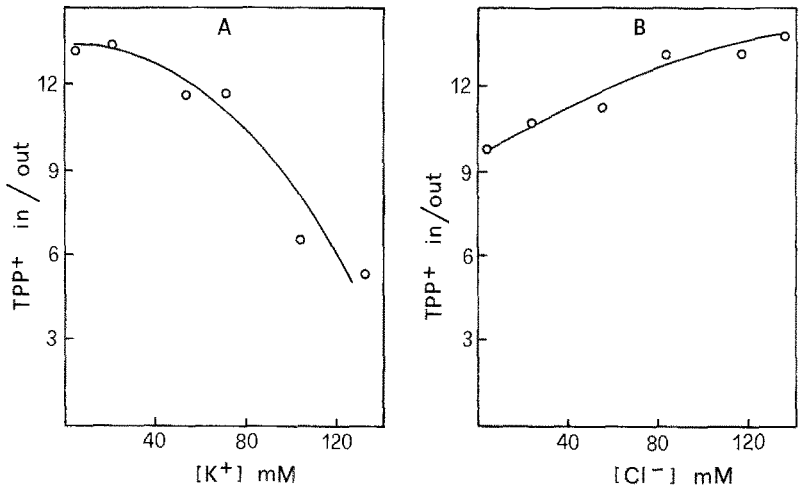

Fig. 7. Effect of extracellular $[K^+]$ and $[Cl^-]$ on TPP^+ accumulation ratio across the plasma membrane. The $[^{14}C]$-TPP^+ accumulation ratio was measured at 30 min in the presence of both 2 μM FCCP and 0.2 mM ouabain to abolish the contribution of $\Delta\psi_m$ and the (Na^+,K^+)ATPase, respectively. (A) $[Cl^-]$ = 133 mM. $[K^+]$ plus $[Na^+]$ = 133 mM. (B) $[K^+]$ = 3 mM, $[Na^+]$ = 130 mM, $[Cl^-]$ plus [gluconate] = 133 mM. All TPP^+ accumulation ratios (mean values of six determinations) were corrected for binding (see text for additional details).

accumulation ratio pattern as a function of extracellular K^+ concentration. It is apparent that the TPP^+ accumulation ratio remains almost constant between 3 and 70 mM extracellular K^+ whereas it greatly decreases at higher K^+ concentrations, in which a correction for a consistent increase in cellular volume had to be introduced. Figure 7B shows that the TPP^+ accumulation ratio decreased slightly but steadily following substitution of extracellular Cl^- by gluconate. Alterations of the extracellular Na^+ concentration by iso-osmotic substitution with choline had no effect on $\Delta\psi_p$ (data not shown). This result, however, was not surprising since it is widely established that the cell plasma membrane is very impermeable to Na^+ ions.

Discussion and Conclusions

The present paper shows that the lipophilic cation TPP^+ is accumulated in cultured human fibroblasts across both the plasma and mitochondrial membranes. As suggested by Felber and Brand (1982), complete abolition of mitochondrial uptake of TPP^+ and careful estimation of its nonspecific binding to cellular components are the prerequisites for a reliable measurement of $\Delta\psi_p$. In this study, the mitochondrial TPP^+ uptake has been abolished

by the uncoupler FCCP, while nonspecific binding has been determined by means of two different experimental procedures leading to the same value. It should be acknowledged, however, that it may be inaccurate to use this value, determined under conditions of maximal depolarization, to correct for binding in all conditions of TPP^+ accumulation. In fact, unspecific binding is dependent on TPP^+ concentration and therefore on the magnitude of both plasma and mitochondrial membrane potentials. However, since determination of binding under polarized conditions is rather complicated in intact cells, we have assumed the potential-independent binding as a minimal value to correct for unspecific binding. The resting value of $\Delta\psi_p$ was 75 mV, which is very similar to that directly measured in human fibroblasts by intracellular microelectrode measurements (Swift and Todaro, 1968; Villereal and Cook, 1978; Moolenaar *et al.*, 1982). These data are clearly in contrast to those of Russel *et al.* (1984) in human fibroblasts, where a value of 109 mV was obtained without any correction for the mitochondrial TPP^+ accumulation.

The finding that the mitochondrial component of TPP^+ accumulation is dependent on the passage number of fibroblast cultures is of particular interest because an earlier report by Hayflick and Moorehead (1961) indicated that diploid human fibroblasts have a limited life-span in culture, an observation which has subsequently been widely confirmed. The decreased TPP^+ accumulation within mitochondria observed at late passage number could be explained on the basis of a decreased number of mitochondria with aging, although there is no consensus on the effect of senescence on mitochondrial number and contribution to the mass of the cell (Harsford, 1983). On the other hand, alterations in membrane lipid composition as well as in the organization and mobility of cell membrane constituents have recently been reported during *in vitro* aging (Yechiel *et al.*, 1986). Changes in membrane lipid composition have a specific effect on membrane integral proteins, including transport proteins (Carruthers and Melchior, 1986), as shown also by the requirement for specific phospholipid and fatty acid types of the successful reconstitution in artificial systems of several mitochondrial membrane associated activities (Krämer and Klingenberg, 1977; Eytan *et al.*, 1976; Rydstrom *et al.*, 1975; Vik *et al.*, 1981). However, the results of experiments performed in senescent animals indicate that mitochondria are substantially undamaged both biochemically and morphologically, but they show a generalized decrease in activity (for a review, see Harsford, 1983). Anyhow it is clear that the age of cell cultures must be carefully checked in any experiments in which normal and pathological cells are compared.

In conclusion, although the TPP^+ accumulation ratio presents difficulties in calculating an absolute value for $\Delta\psi_m$ and $\Delta\psi_p$, our findings are consistent with its ability to detect changes in both membrane potentials in fibroblasts. Furthermore, TPP^+ accumulation under appropriate conditions could be a

useful indicator of mitochondrial involvement in cellular events, such as, for instance, viral infection (Landini and Rugolo, 1985).

Acknowledgments

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