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UNDESIRABLE FEATURE OF SAFRANINE AS A PROBE FOR MITOCHONDRIAL MEMBRANE POTENTIAL

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SUMMARY: This communication describes experiments showing that safranine, at the concentrations usually employed as a probe of mitochondrial membrane potential, causes significant undesirable side effects on Ca^{2+} transport by liver mitochondria. The major observations are: (i) safranine potentiates the spontaneous Ca^{2+} release from liver mitochondria induced by phosphate or acetoacetate. This is paralelled by potentiation of the release of state-4 respiration and of the rate of mitochondrial swelling, indicating a generalized effect of the dye on the mitochondrial membrane; (ii) the efflux of mitochondrial Ca^{2+} stimulated by hydroperoxide is irreversible in the presence of safranine even if membrane stabilizers such as Mg^{2+} and ATP are present. It is concluded that the use of safranine to monitor the changes in membrane potential during Ca^{2+} transport by mitochondria should be avoided or special care be taken.

Safranine, a lipophylic positively charged dye, has been frequently employed as a probe of the mitochondrial membrane potential ($\Delta\psi$), in view of the spectral absorption change that occurs when it staks to the membrane of energized mitochondria (1,2). It was first shown by Åkerman and Wikström (3) that this spectral shift of safranine is linearly related to the magnitude of the membrane potential in the range 50 to 170 mV. It is well established that safranine moves from the outer to the inner space under the influence of $\Delta\psi$ and distributes at electrochemical equilibrium attaining a millimolar concentration in the matrix in the presence of a $\Delta\psi$ of about 180 mV, negative inside (4).

A number of quantitative problems were recently raised concerning the use of the safranine spectral shift for the determination of $\Delta\psi$ (4), involving: (i) the relationship between the extent of safranine absorbance change and the amount of mitochondrial protein; (ii) the linearity of the dye response to $\Delta\psi$ changes; and (iii) the time response of the dye.

Abbreviations: EGTA, ethylene glycol bis (β-aminoethyl ether) N, N'- tetraacetic acid; Hepes, 4-(α-hydroxyethyl-1-piperazine -ethane-sulfonic acid; $\Delta\psi$, transmembrane electrical potential ; TPP^+, tetraphenylphosphonium; RLM, rat liver mitochondria.

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In addition, it was shown that the uptake and binding of safranine result in mitochondrial damage with respect to the degree of energy coupling, rate of electron transport and ADP-stimulated respiration (4).

The experiments described in this communication show that safranine, at the concentrations usually employed as a probe of the mitochondrial membrane potential, also causes significant undesirable side effects on mitochondrial functions associated with Ca^{2+} accumulation under certain experimental conditions.

EXPERIMENTAL PROCEDURES

Liver mitochondria were isolated by a conventional differential centrifugation method (5) from overnight fasted female Wistar-strain rats weighing aproximately 250g. Mitochondrial protein was determined according to Kies and Murphy (6). All the incubations were carried out in thermostatized glass chambers or cuvettes at 30°C. Changes in Ca^{2+} concentrations in the suspending medium were followed using a Ca^{2+} -selective electrode (Radiometer, F2112), calibrated with internal standards added to each reaction medium as in ref.7. Oxygen consumption was measured with a Clark electrode. Mitochondrial swelling was estimated by light scattering changes at 520 nm in an Aminco Bowman Spectrophotofluorometer. Changes in membrane potential were determined with a TPP^{+}-electrode made in our laboratory according to Kamo et al (8). The Ca^{2+} flux, $\Delta\psi$ and swelling measurements carried out in the presence of safranine were corrected graphically to compensate the interference of the dye on the calibrations. Safranine O (obtained from Aldrich Chemical Co.) was dissolved in water at a stock concentration of 10 mM.

RESULTS

Potentiation of spontaneous Ca^{2+} release from liver mitochondria by safranine

It is well documented that Ca^{2+} accumulation by liver mitochondria incubated in phosphate-containing media in the absence of exogenous Mg^{2+} and ADP or ATP is followed by a spontaneous release of the accumulated Ca^{2+} after a variable lag of up to 20 min. (c.f.ref.9 for review). This spontaneous Ca^{2+} discharge is preceded by large amplitude mitochondrial swelling and collapse of the membrane potential (9). This phenomenon is potentiated by several agents, such as: phosphoenolpyruvate (10); atractylate (11); the NAD(P)H oxidants acetoacetate oxaloacetate and hydroperoxide (7,12); long-chain acyl-CoA (13); etc. The experiment in Fig.1 shows the onset of Ca^{2+} uptake and release and the rates of respiration and swelling of rat liver mitochondria incubated in medium containing 30 nmol $Ca^{2+}.mg^{-1}$ and 2.0 mM phosphate, in the presence or absence of different concentrations of safranine (traces a,b,c and d). It can be seen that in the absence of safranine Ca^{2+} uptake was followed by a lag of about 13 min. before the initiation of fast Ca^{2+} release. During this period, a progressive increase in the rate of respiration and a continuous increase in mitochondrial swelling also occurred. The lag period between Ca^{2+} uptake and release became shorter

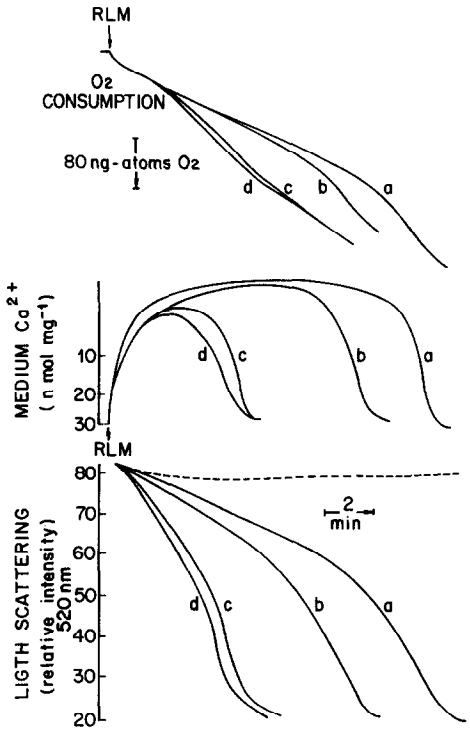

Figure 1. Effect of safranine concentration on Ca^{2+} retention, rate of respiration and swelling of rat liver mitochondria. The reactions were initiated by the addition of mitochondria (1 mg protein) to a medium (1 ml) containing 125 mM sucrose, 65 mKCl, 3.0 mM Hepes (pH 7.1), 5.0 µM rotenone, 2.0 mM phosphate, 2.0 mM succinate and 30 nmol Ca^{2+} in the absence (a) or presence of 5 µM(b), 10 µM(c) or 20 µM(d) safranine. The broken line represents one experiment carried out in the absence of both Ca^{2+} and safranine.

with increasing safranine concentrations in the medium. The release of state-4 respiration and the rate of mitochondrial swelling were also accelerated by safranine.

The experiment in Fig.2 shows that the effect of acetoacetate in accelerating the release of state-4 respiration and decreasing the period of Ca^{2+} retention by liver mitochondria was also potentiated by safranine. In this experiment the phosphate concentration (0.3 mM) was much smaller than that used in Fig.1 and Ca^{2+} was retained by mitochondria as long as oxygen was present in the medium (trace a). In the presence of acetoacetate, however, Ca^{2+} release initiated before anaerobiosis was attained (trace b). Safranine, at a concentration of 10 µM, which is usually employed to monitor $\Delta\psi$, significantly decreased the period of Ca^{2+} retention by mitochondria (trace c), when compared with the experiment where only acetoacetate was present (trace b).

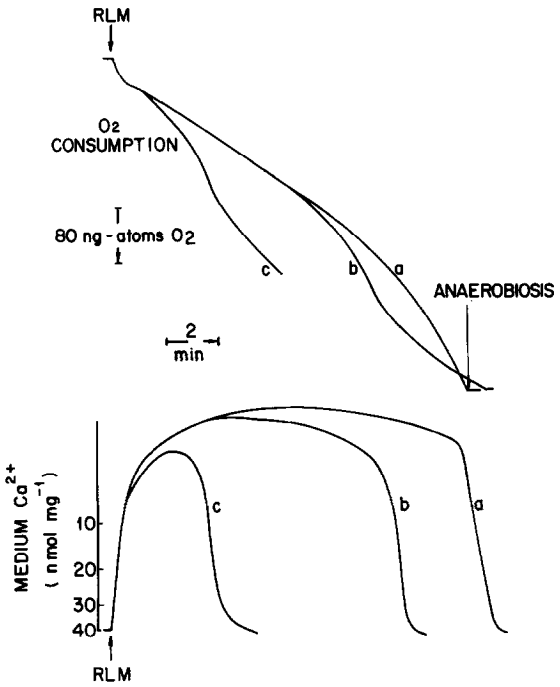

Figure 2. Potentiation by safranine of acetoacetate-induced release of state 4 respiration and Ca^{2+} from liver mitochondria. The reactions were initiated by the addition of mitochondria (1mg) to a medium (1ml) containing 125 mM sucrose, 65 mM KCl, 3.0 mM Hepes (pH 7.1), 5.0 µM rotenone 0.3 mM phosphate 2.0 mM succinate and 40 nmol Ca^{2+} (a), plus 1.0 mM acetoacetate (b), or 1.0 mM acetoacetate plus 10 µM safranine (c).

Effect of safranine on Ca^{2+} flux in medium containing Mg^{2+} and ATP

The effect of safranine on Ca^{2+} flux was also studied in mitochondria suspended in medium containing Mg^{2+}, ATP and acetate instead of phosphate as permeant anion. Panel A of Fig.3 shows that in the presence of Mg^{2+} and ATP, Ca^{2+} (100 nmol.mg^{-1}) was accumulated and retained by mitochondria. A $\Delta\psi$ of about 175 mV and a constant rate of state-4 respiration were maintained throughout the experiment. Under these conditions only a small decrease in the rate of both Ca^{2+} uptake and formation of $\Delta\psi$ and a small increase in the rate of respiration were caused by safranine (broken lines). The dotted line in Fig.3A shows for comparison, the values of $\Delta\psi$ in the absence of both safranine and Ca^{2+} (EGTA present from the beginning). Panel B shows that in the presence of ATP and Mg^{2+} a small amount of hydroperoxide (50 nmol) caused a transient increase in extramitochondrial Ca^{2+} concentration just before the completion of Ca^{2+} uptake. This was paralelled by a transient increase in the rate of respiration and a decrease in $\Delta\psi$. In agreement with previous findings of this laboratory (14), after this

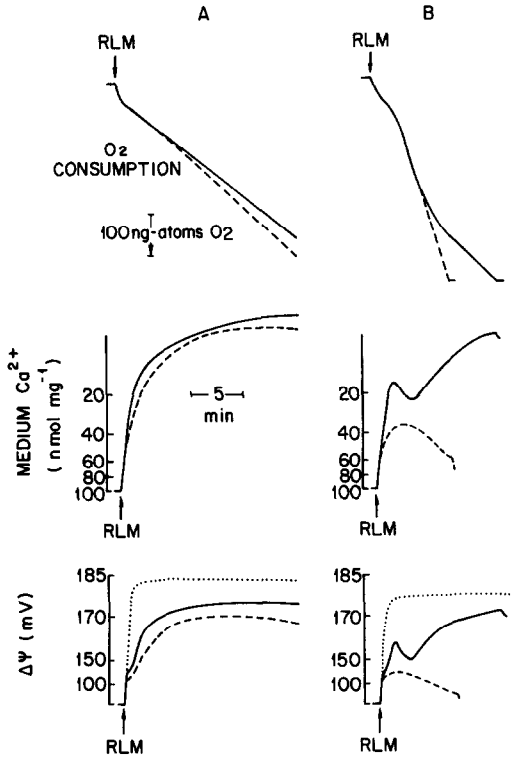

Figure 3. Effect of safranine on Ca^{2+} transport by rat liver mitochondria in the absence (A) or presence of hydroperoxide (B) in media containing Mg^{2+} and ATP. The reactions were initiated by the addition of mitochondria (1 mg) to a medium (1 ml) containing 125 mM sucrose, 65 mM KCl, 3.0 mM Hepes (pH 7.1), 10 mM acetate, 30 mM TPP^{+} and 100 nmol Ca^{2+} in the absence (solid lines) or presence of 10 μM safranine (broken lines). The dotted lines represent experiments carried out in the presence of 1.0 mM EGTA in the absence (A) or in the presence of 10 μM safranine (B).

transient increase in extramitochondrial Ca^{2+} concentration observed during hydroperoxide metabolism (12), the cation was retained by mitochondria until anaerobiosis was attained. However, when these experiments were carried out in the presence of 10 μM safranine, a completely different pattern was observed. It can be seen in Fig.3B that $\Delta\psi$ attained a maximum value of about 120-130 mV and declined slowly. As a consequence Ca^{2+} was only partially accumulated and not retained and a fast rate of respiration was maintained until the medium oxygen was exausted. In the absence of both Ca^{2+} (EGTA present) and hydroperoxide, a high $\Delta\psi$ was built up and maintained even in the presence of safranine (Fig.3B,dotted line).

DISCUSSION

The present study shows that safranine, in the range of concentrations usually employed as a probe of the mitochondrial membrane

potential, causes significant undesirable side effects on Ca^{2+} transport by mitochondria under certain experimental conditions. For instance, the experiments in Figure 1 and 2 show that safranine strongly potentiates the effect of phosphate and acetoacetate in promoting mitochondrial damage caused by Ca^{2+} accumulation when liver mitochondria were preincubated in the absence of Mg^{2+} and ATP (9). On the other hand, the experiments in Fig.3A show that when Ca^{2+} flux was studied in mitochondria suspended in medium containing Mg^{2+} and ATP the side effect of safranine alone was very small even at the high Ca^{2+} load employed (100 nmol mg^{-1}). However, under these experimental conditions, safranine has a significant effect in modifying the pattern of alterations in Ca^{2+} flux, membrane potential and respiration caused by hydroperoxide. An analysis of the effects of safranine on Ca^{2+} flux, $\Delta\psi$, mitochondrial swelling and respiration indicates a generalized action of the dye, possibly related to its lipophylic nature, on the mitochondrial membrane. The effect of safranine is much more pronounced under conditions in which Ca^{2+} transport *per se* is deleterious to mitochondria. In the presence of EGTA or when Ca^{2+} transport was studied in medium containing ATP plus Mg^{2+}, the side effects of safranine alone in mitochondria were absent or not detected with the methodology employed in this study. It can be concluded from these results that safranine can be included in the list of agents that sensitise mitochondria to the deleterious effect of Ca^{2+} and therefore cause spontaneous discharge of this cation from the organelle (9).

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